NODULAR LYMPHOCYTE PREDOMINANT HODGKIN LYMPHOMA – STUDY OF IMMUNOARCHITECTURAL PATTERNS AND THE USEFULNESS OF PD-1 AND CD57 IN THE DIFFERENTIAL DIAGNOSIS WITH T CELL/ HISTIOCYTE RICH LARGE B CELL LYMPHOMA

A DISSERTATION SUBMITTED IN PART FULFILMENT OF THE REGULATION FOR THE AWARD OF THE DEGREE OF M.D. PATHOLOGY

BRANCH III

THE TAMIL NADU DR. M.G.R. UNIVERSITY

CHENNAI, TAMIL NADU

APRIL-2016

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CERTIFICATE

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Dr. Banumathi Ramakrishna, MD, MAMS
Professor and Head,
Department of Pathology,
Christian Medical College,
Vellore.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Principal,
Christian Medical College,
Vellore.

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Dr. Marie Therese Manipadam, MD, DNB
Professor,
Department of Pathology,
Christian Medical College,
Vellore.

CERTIFICATE

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I have independently reviewed the literature, standardized the data collection methodology and carried out the evaluation towards completion of the dissertation.

Dr. Preethi Morais,
PG Registrar,
Department of Pathology,
Christian Medical College,
Vellore.

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Dr. Banumathi Ramakrishna, MD, MAMS,

Professor and Head,

Department of Pathology,

Christian Medical College,

Vellore.

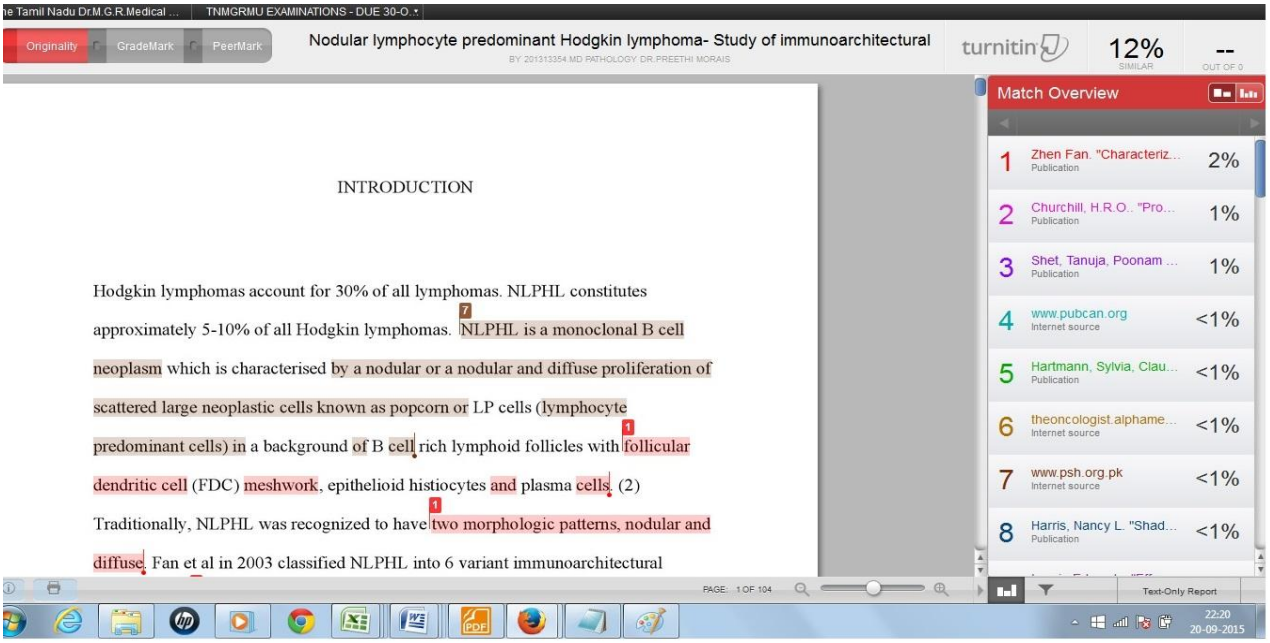
PROFESSOR AND HEAD,
DEPARTMENT OF PATHOLOGY,
Christian Medical College,
IDA Scudder Road, Vellore - 632 004. INDIA.

Dr. Alfred Job Daniel, D Ortho, MS (Ortho), DNB (Ortho),

Principal,

Christian Medical College,

Vellore.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INTRODUCTION
Hodgkin lymphomas account for 30% of all lymphomas. NLPHL constitutes approximately 5-10% of all Hodgkin lymphomas. NLPHL is a monoclonal B cell neoplasm which is characterised by a nodular or a nodular and diffuse proliferation of scattered large neoplastic cells known as popcorn or LP cells (lymphocyte predominant cells) in a background of B cell rich lymphoid follicles with follicular dendritic cell (FDC) meshwork, epithelioid histiocytes and plasma cells. (2) Traditionally, NLPHL was recognized to have two morphologic patterns, nodular and diffuse. Fan et al in 2003 classified NLPHL into 6 variant immunoarchitectural
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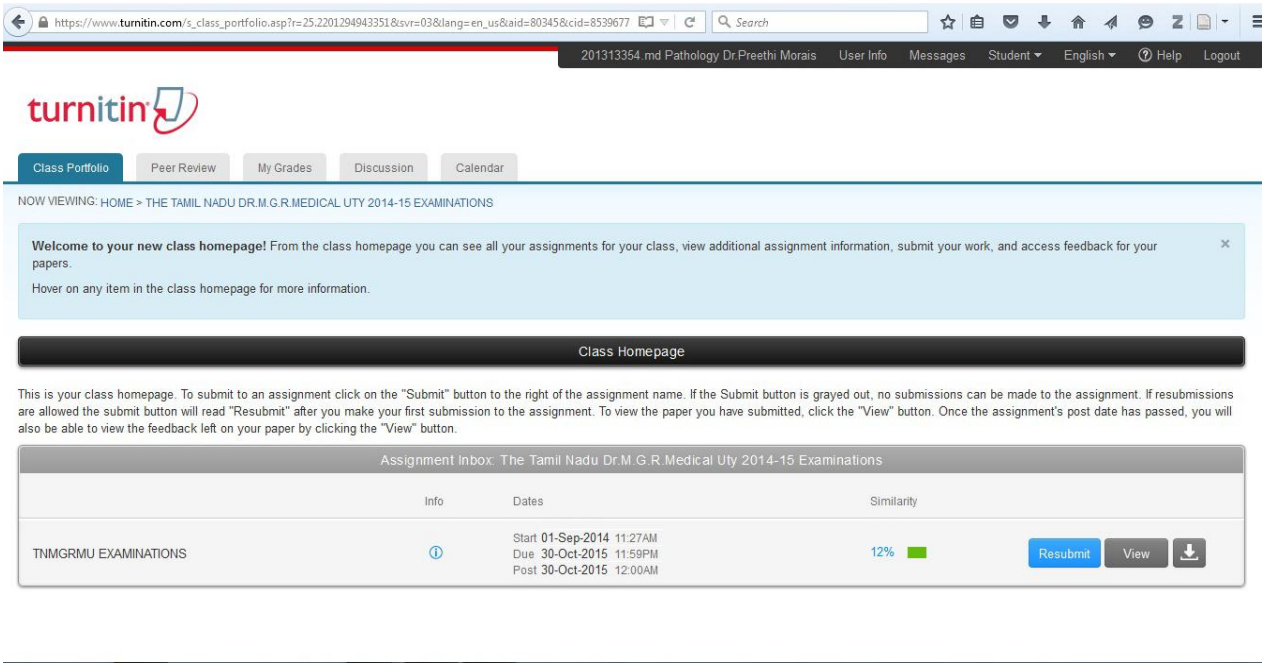
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Dr. Preethi Morais
PG Registrar
Department of General Pathology
Christian Medical College, Vellore 632 004

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Nodular lymphocyte predominant Hodgkin lymphoma- study of immuno architectural patterns and usefulness of PD1 and CD57 immunohistochemistry in differential diagnosis from T cell rich B cell lymphoma.
Dr. Preethi Morais, PG Registrar, General Pathology, Dr. Marie Therese Manipadam, General Pathology, Dr. Auro Viswabandya , Haematology, Mrs. Grace Rebekah. J, Biostatistics, CMC, Vellore.

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Dear Dr. Preethi Morais,

I enclose the following documents:-

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With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Cc: Dr. Marie Therese Manipadam, General Pathology, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

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Dr. Preethi Morais, PG Registrar, General Pathology, Dr. Marie Therese Manipadam, General Pathology, Dr. Auro Viswabandya , Haematology, Mrs. Grace Rebekah. J, Biostatistics, CMC, Vellore.

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Yours sincerely,

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ACKNOWLEDGEMENTS

Though this dissertation is an individual work, I would never have been able to complete it without the help, motivation and guidance of many people. Firstly, I am immensely grateful to my guide and mentor Dr. Marie for her untiring patience, constant support, encouragement and dedication to assist me during the entire period of my study. I would like to thank Dr. Banumathi Ramakrishna for her support and faith in our whole batch. I thank Mrs. Gowri and Mrs. Kavitha for their assistance for the statistical analysis. I thank the lab technicians and staff, especially Mr. Vijay Kumar, Mrs. Annie, Mr. Daniel and Mr. Sam for their support and ever obliging nature to help. I thank my friends who have been with me throughout this period. Last but not the least, I owe a ton to my family and Vincent for all the moral support, faith and constant prayers for me during this entire phase of my life.

CONTENTS

INTRODUCTION

INTRODUCTION

Hodgkin lymphomas account for 30% of all lymphomas. Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) constitutes approximately 5-10% of all Hodgkin lymphomas. NLPHL is a monoclonal B cell neoplasm which is characterised by a nodular or a nodular and diffuse proliferation of scattered large neoplastic cells known as popcorn or LP cells (lymphocyte predominant cells) in a background of B cell rich lymphoid follicles with follicular dendritic cell (FDC) meshwork, epithelioid histiocytes and plasma cells. (1) Traditionally, NLPHL was recognized to have two morphologic patterns, nodular and diffuse. Fan et al in 2003 classified NLPHL into 6 variant immunoarchitectural patterns. The recognition of these patterns is diagnostically significant and would aid the clinician in the treatment and follow up. (2) The distinction between the diffuse types of NLPHL from T cell histiocyte rich large B cell lymphoma (THRLBCL) has been a controversy due to the overlapping morphological and immunohistochemical features. THRLBCL is a variant of diffuse large B cell lymphoma(DLBCL) [B cell non Hodgkin lymphoma] in which only scattered malignant cells are present in a background of T lymphocytes, usually with single or small clusters of histiocytes. (3) The tumour cells may resemble the LP cells. Since there is a grey area between NLPHL – diffuse pattern and THRLBCL and the distinction between the two can be difficult, WHO 2008 agreed that the diagnosis of THRLBCL should be restricted to primary cases and that occurrence of relapse of NLPHL with a partially or entirely diffuse pattern should be called ‘diffuse LPHL’ or NLPHL- THRLBCL like’. The importance in differentiating

NLPHL and TCRLBCL is because they are two distinct entities and both have distinctly different biologic behaviour and different therapeutic regimens. There have been few studies which have assessed the diagnostic utility of immunomarkers Programmed death-1 (PD1) in comparison with Cluster of Differentiation 57 (CD57). (4)(5) Our study aims at classifying NLPHL cases from our institution into the variant immunoarchitectural patterns as described by Fan et al. We also intend to study the frequency of T cell rosettes staining for immunohistochemical markers PD1 and CD57 in the cases of NLPHL and THRLBCL and assess their utility in the diagnosis of NLPHL and differentiation of NLPHL from THRLBCL.

JUSTIFICATION

JUSTIFICATION

1. The cases diagnosed as NLPHL in our institution needs to be sub classified into the variant patterns since they have a prognostic significance. (2)
2. There have been very few International studies (2)(5)(6) and only one study from India (7) that have analysed the immunoarchitectural patterns of NLPHL.
3. NLPHL and THRLBCL resemble each other both morphologically as well as immunohistochemically, but are two distinct entities and both have different therapeutic regimens. We selected 2 markers, PD1 and CD57 to assess their utility in the differentiation of NLPHL to THRLBCL. There have been only two studies till date that have compared these two markers in the diagnosis of NLPHL. (4)(5)

AIMS

AIMS

In our dissertation we aim to study the histomorphological features of NLPHL, classify it according to the variant immunoarchitectural patterns as described by Fan et al (2) and analyse the frequency of each pattern in an Indian population. We also aim to study the role of the immunohistochemical markers PD1 and CD57 in the differentiation of NLPHL from THRLBCL.

OBJECTIVES

OBJECTIVES

1. To do a detailed histo-morphological assessment of the cases diagnosed as NLPHL over a ten year period (January 2003 to December 2013).
2. To sub classify our cases into the variant immunoarchitectural patterns.
3. To assess and compare the utility of PD1 & CD57 in the differential diagnosis of THRLBCL from NLPHL.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

INTRODUCTION

Hodgkin lymphoma (HL) was described in 1832 by Thomas Hodgkin (8), but his work went unnoticed for many years. In 1865, Samuel Wilks published a paper describing a series of cases with lymphatic gland and splenic enlargement and named the condition *Hodgkin's disease* in honour of his predecessor, Thomas Hodgkin. The histopathologic features of Hodgkin lymphoma was first described by Theodore Langhans in the year 1872 and WS Greenfield in the year 1878. Subsequently, Dorothy Reed and Carl Sternberg gave a detailed description of the presently called Reed Sternberg (RS) cells, in 1898 and 1902 respectively.

Hodgkin lymphoma has gone through several changes since it was first described. Table.1 summarizes the changes that have taken place since it was first discovered.

Table.1: Evolution of the classification of Hodgkin lymphoma

1944	JACKSON AND PARKER CLASSIFICATION	• Granuloma • Paragranuloma • Sarcoma
1966	LUKE AND BUTLER CLASSIFICATION	• Lymphocytic and/or histiocytic, nodular • Lymphocytic and/or histiocytic, diffuse • Nodular sclerosis • Mixed cellularity • Diffuse fibrosis • Reticular
1966	RYE'S CLASSIFICATION	• Lymphocyte predominance • Nodular sclerosis • Mixed cellularity • Lymphocyte depletion
1994	REAL CLASSIFICATION	• Lymphocyte predominant Hodgkin lymphoma • Classical Hodgkin lymphoma ➢ Nodular sclerosis HL ➢ Lymphocyte rich classical HL ➢ Mixed cellularity HL ➢ Lymphocyte depleted HL
2001 & 2008	WHO CLASSIFICATION	• Nodular lymphocyte predominant Hodgkin

	lymphoma • Classical Hodgkin lymphoma ➢ Nodular sclerosis HL ➢ Lymphocyte rich classical HL ➢ Mixed cellularity HL ➢ Lymphocyte depleted HL

(3)(9)

As mentioned above, Hodgkin lymphoma is broadly classified into two types: NLPHL and classical Hodgkin lymphoma (CHL). The similarities between the two are that there is a paucity of neoplastic cells in a background rich in inflammatory cells.

NLPHL is the rarer subtype of Hodgkin lymphoma and constitutes 5%-10% of the cases. (10) The first known description of NLPHL dates back to 1936, when Rosenthal observed that the overall survival of patients with HL was directly related to the lymphocyte proliferation within the lymph nodes. In 1994, the revised European-American lymphoid (REAL) malignancy classification described this lymphoma in detail and described the immunophenotype of this malignancy in detail. (11)

NLPHL is a different disease when compared to classical Hodgkin lymphoma. The neoplastic cells in NLPHL are the lymphocyte predominant (LP) / popcorn cells in contrast to the RS cells in HL. The other important and key feature differentiating the two is their immunophenotype, a more detailed description of which is described subsequently.

DEFINITION:

The WHO 2008 defines NLPHL as a nodular or a nodular and diffuse proliferation of scattered large neoplastic cells known as popcorn or LP cells – formerly called L&H cells for lymphocytic and/or histiocytic Reed Sternberg cell variants. These cells reside in a background of B cell rich lymphoid follicles with follicular dendritic cell meshwork, epithelioid histiocytes and plasma cells. At least a partial nodular pattern is required for the diagnosis of NLPHL to be made. (1)

EPIDEMIOLOGY:

NLPHL constitutes 5-10% of all Hodgkin lymphomas. There have been studies which have described two peaks, the first among children and adolescents and the second among adults >40years. (12)(13) Other studies have reported the median age at presentation of NLPHL to be 30-50 years and among children was 13 years. (14)(15)(16)There is a striking male preponderance (even among children) with the male:female ratio being 3:1.

In a study conducted by Morton et al., the incidence rate of NLPHL among Americans was found to be as low as 0.08/1, 00,000 person years. The incidence rate ratio among Asian males to females was found to be lower. They found higher rates among white males when compared to any sex or race. (17)

Arora et al., conducted a retrospective study in an Indian cohort over a 10 year period (2001-2010) and analysed 5115 patients who were diagnosed as lymphoma. They found that majority of their cases were non Hodgkin lymphoma (NHL) constituting 78.7% and Hodgkin lymphoma constituting only 21.3%. Of the Hodgkin lymphomas,

NLPHL constituted 4%, which was in keeping with the incidence rates of studies conducted in China and the West. (18)(3)(19) The mean age at diagnosis for NLPHL was found to be 33 years with the male: female ratio being 2.6. (20)

Patkar et al., analysed 451 cases of HL from an Indian population (predominantly Western Indian) over a period of 2.5 years and found 11.97% (54 cases) to be NLPHL. These cases of NLPHL ranged from 5 to 78 years of age with the mean age at diagnosis being 35 years. The sex predominance was similar to the other studies. (21)

SITES OF INVOLVEMENT:

NLPHL usually presents with peripheral/superficial lymphadenopathy which is in contrast to CHL where the central (mediastinal / hilar) lymph nodes are involved. The most commonly involved lymph node groups include cervical lymph nodes and axillary lymph nodes. The spread of disease is non-contiguous. (22) The involvement of central group of lymph nodes and extra nodal disease is rare with splenic involvement being 10-15%, liver involvement <10% and bone marrow and lung involvement being <5%. (13) There have been rare reports of NLPHL involving the colon, breast (20) and appendix (23).

CLINICAL FEATURES:

The most common clinical symptom of this disease is localised painless peripheral lymphadenopathy. There has found to be a time lag between the first appearance of lymph nodes to the confirmation of NLPHL. (24) These patients usually present at an early stage, stage IA or IIA disease. The classic B symptoms of classic Hodgkin

lymphoma, which include fever, night sweats and loss of weight, are seen less frequently.

MORPHOLOGY:

A diagnosis of NLPHL can be made based on several distinct morphological features, the most important and essential among them being the presence of at least a single nodule in a lymph node biopsy.

Morphologically, NLPHL is characterised by scanty neoplastic cells in a predominantly reactive background. Classically, the neoplastic LP cells or popcorn cells have a single large folded or multilobated nucleus, several small basophilic nucleoli, thin nuclear membranes and moderate to scant cytoplasm. (3)(10) These cells reside in a background of FDC meshwork admixed with lymphocytes (predominantly B cells), epithelioid histiocytes and plasma cells.

IMMUNOHISTOCHEMISTRY:

Immunohistochemically, the *neoplastic LP cells* express positivity for CD20, BCL6, CD75, CD79a and CD45.

Many of the cases show positivity for J-chain. EMA positivity is seen in 50% of the cases. Nuclear transcription factors such as OCT2, BOB1 and activation induced diaminase (AID) are positive. Immunoglobulin light and heavy chains including IgD are strongly positive. CD15 and CD30 are frequently negative. However, few of the cases show CD30 positive large cells which are probably reactive immunoblasts.

The neoplastic LP cells are surrounded by *T-cell rosettes* which are positive for PD1, CD57and CD3.

The reactive background is composed of follicular dendritic cells, small B-cells and T-cells.

The *follicular dendritic cells* are positive for CD21 and CD23. CD23 was found to be a better marker than CD21 in highlighting the FDC meshwork.(7)

The *background B-cells* are CD20 positive and are useful in classifying NLPHL into the variant immunoarchitectural patterns.

The *background T-cells* expresses PD1, CD57, IRF4/MUM1, BCL6, cMAF and CD134.

PATTERNS DESCRIBED IN NLPHL:

Traditionally, NLPHL was morphologically characterised by a nodular or nodular and diffuse pattern. But in 2003, Fan et al., after analysing 137 biopsy samples from 118 patients diagnosed as NLPHL, described 6 variant immunoarchitectural patterns of NLPHL. These include:

A) ***"Classic" nodular pattern, B cell rich***
B) ***Serpiginous/interconnected nodular pattern***
C) ***Nodular with prominent extranodular L&H cells***
D) ***Nodular with T- cell-rich background***
E) ***Diffuse pattern (T-Cell-Rich B-Cell Lymphoma-like)***
F) ***(Diffuse), "Moth-eaten" with B-cell-rich background***

A) ***"Classic" nodular pattern, B cell rich:***

This pattern has been described as the prototype of NLPHL. It is characterised by nodules within which lie scattered popcorn cells. The nodules are predominantly composed of small non neoplastic B cells. On a rare occasion, the popcorn cells may be found outside the nodules.

B) ***Serpiginous/interconnected nodular pattern:***

This pattern is composed of irregularly shaped, interconnected nodules arranged in a serpiginous pattern. Apart from this feature, it is identical to the "classic" nodular pattern, B cell rich.

C) ***Nodular with prominent extranodular L&H cells:***

This pattern is composed of poorly circumscribed reactive nodules with more of T-cells than B-cells within them. These nodules are surrounded by many popcorn cells which are set in a background of T-cells and can be distributed in an inter-nodular or band like fashion around these nodules. They can also lie adjacent to or merge with the diffuse T-cell rich areas. These extranodular popcorn cells are neither associated with a follicular dendritic cell meshwork nor CD 57 positive T-cell rosettes around them.

D) Nodular with T- cell-rich background:

This type is composed of T-cell rich nodules set in a T-cell rich background. These nodules usually display a follicular dendritic cell

meshwork, despite the fact that the FDC meshwork gets attenuated with fewer B-cells. The popcorn cells are surrounded by CD 57 positive T-cell rosettes.

E) Diffuse pattern (T-Cell-Rich B-Cell Lymphoma-like)

This pattern is defined by the presence of at least a single nodule with popcorn cells within, in a diffuse background of reactive T cells and with interspersed popcorn cells in the background. Here there is a loss of follicular dendritic cell meshwork (along with loss of CD 57 positive T lymphocytes). The important feature in this pattern, to differentiate from THRLBCL, is the presence of at least one nodule in the biopsy.

F) (Diffuse), "Moth-eaten" with B-cell-rich background

This pattern is composed of a B-cell rich background with interspersed popcorn cells, which are rimmed by T-lymphocytes. Fan et al., have described this pattern as a diffusely expanded single nodule of a classic nodular pattern. The follicular dendritic meshwork is maintained and it is the T-lymphocytes, which do not take up the CD 20 stain that impart a "moth eaten appearance".

There are certain other morphologic features which can be associated with NLPHL, which include:

- Small germinal centres
- Sclerosis

- Granulomas
- Progressive transformation of germinal centres

Small germinal centres:

Small germinal centres can be seen within the nodules of NLPHL. They are more commonly seen at the periphery of the nodules containing the popcorn cells.
Fan et al., analysed 137 biopsy samples of 118 patients, and found 15% of their cases (17 of 118 patients) to have small germinal centres. On analysing the significance of these small germinal centres, they found that they do not have any correlation with clinical characteristics. (2)

Sclerosis:

Varying types of sclerosis can be seen associated with NLPHL. These include: broad bands of sclerosis, extensive hyaline type of sclerosis and nodular masses of sclerosis. Fibrosis is also seen at the rim of the nodules. Prominent sclerosis need not necessarily be associated with a previous history of trauma in the form of needle aspirations or previous biopsies.
Studies by Fan et al., have shown that prominent sclerosis has been associated with disease recurrence, but multivariate analysis did not show this to be an independent predictor of disease recurrence. (2) They demonstrated prominent sclerosis in 44% of patients (out of 118 patients) with disease recurrence and in 7% (out of 118 patients) of patients without recurrence.

Granulomas:

NLPHL is usually not associated with granulomas. However, histiocytes may be seen scattered forming small granulomas at the periphery of the nodules. (24)

Progressive transformation of germinal centres (PTGC):

This is a benign reactive condition, first described by Lennert and Muller-Hermelink in 1975. The exact etiology and pathogenesis is unknown. In the quest to discover the origin of PTGC, various proposals had been put forth, of which PTGC being a pre-neoplastic stage of NLPHL is one of them. (25)(26)(27)

Histologically, PTGC is characterised by the migration or falling of small mantle zone lymphocytes into the germinal centres with progressive accumulation, eventually expanding the germinal centres into enlarged and irregularly shaped nodules with blurred mantle zones. The feature to differentiate this from NLPHL is the absence of LP cells. Table.2 summarizes the differences between NLPHL and PTGC.

Table.2: Comparison of NLPHL and PTGC.

NLPHL	PTGC
Young males	Young males
Usually entire lymph node involved	Partial lymph node involvement
Complete effacement of architecture by one or a combination of the 6 immunoarchitectural patterns	One or two enlarged nodules in a background of reactive follicular hyperplasia
Interfollicular area involved	Interfollicular area uninvolved
LP cells present	LP cells absent

Since there were many proposals that PTGC is a precursor of NLPHL, Ferry et al., did a follow up study of 5 patients with florid PTGC and proved that PTGC is not associated with NLPHL. (28)

SIGNIFICANCE OF CHARACTERISATION OF NLPHL INTO VARIANT PATTERNS:

From the published data, there have been four studies till date that have taken into consideration the variant patterns of NLPHL. (2)(6)(5)(7)

Fan et al., were the pioneers in classifying NLPHL into 6 variant immunoarchitectural patterns. They found that pattern C and pattern E had some clinical significance.

NLPHL with prominent extranodular LP cells were found to be associated with progression or an early evolution to a higher pattern (diffuse pattern).

The diffuse pattern was found to be associated with cases that recurred. Another interesting finding Fan and colleagues noticed was that disease progression was associated with more diffuse areas. Definite results could not be concluded due to the short follow up period and retrospective nature of their study. However, documentation of the presence of diffuse areas and their amount would be useful in the management of the patient. (2)

Hartmann et al., assessed the frequency and prognostic implications of the variant patterns of NLPHL. They included 423 biopsy samples and classified them into tumour rich cases (10 cases), typical NLPHL – patterns A and B (308 cases) and histopathologic variants – patterns C, D, E and F (105 cases). They formulated a scoring system to classify patients into 3 risk groups – high, intermediate and low, based on the histopathologic and clinical features. The progression free survival (PFS) and overall survival (OS) were assessed. The 5 year PFS/OS for the low risk patients was found to be 95.2%/ 98.7%, for intermediate risk patients 87.5%/96.2% and for the high risk patients was 68.7%/88.3%. They found that the histopathologic variants were associated with poorer outcome, advanced disease and was found to be an independent risk factor for relapse. They also concluded that the higher risk group patients may be candidates for novel treatment strategies.(6)

Churchill et al., performed a retrospective analysis on 67 cases of NLPHL, 6 cases of THRLBCL and 5 cases of lymphocyte rich classic Hodgkin lymphoma (LRCHL) and assessed the expression of an immunohistochemical marker, PD1 in comparison with CD57 in the variant patterns of NLPHL. They concluded that PD1 was a superior marker than CD57 in the nodular patterns of NLPHL (i.e., “Classic” nodular pattern,

B cell rich, Serpiginous/interconnected nodular pattern, Nodular with T- cell-rich background).

The expression of PD1 positivity outside the nodules, surrounding the extranodular LP cells was seen in 66% of cases, in contrast to 33% of cases using CD57. This is valuable information as identification of pattern C is clinically important since it is associated with a higher risk of progression, and hence close follow up can be done for these patients. Another finding in their study was that there was gradual loss of expression of PD1 from the nodular to diffuse areas. (5)

Shet et al., devised a three tier scoring system to quantify the variant patterns in a patient and help the treating physician in understanding the extent of variation. They assessed 5 parameters and stratified 72 patients into two groups, those with a score of </=6 (42 patients) and >6 (30 patients). The five parameters used in this scoring system were: 1) percentage of nodularity, 2) extranodular LP cells, 3) ratio of T cells vs B cells, 4) types of nodules and 5) loss of dendritic meshwork.

They found that the patients with a lower score had a better survival than those with a higher score. For patients with a score </=6, the 5 year overall survival (OS) was 100%, the median disease free survival (DFS) was 133.6 months and 5 year DFS was 90%. For patients with a score >6, the OS was 87%, median DFS was 35 months and 5 year DFS was 20%.

PROGNOSIS:

NLPHL is an indolent disease with an overall good prognosis. The long term remission rate of NLPHL is greater than 90%, provided they are given standard

treatment regimens. (26) There is limited literature describing the predictive markers of NLPHL, the probable reason being its rarity and overall good prognosis. Porrata et al., did a study including 103 consecutive NLPHL patients at Mayo Clinic between 1974-2010 and demonstrated that the peripheral blood absolute lymphocyte count/ absolute monocyte count ratio at diagnosis (ALC/AMC-DX) was a predictor of superior survival in NLPHL and CHL. (30)

This disease is associated with frequent late relapses and an increased susceptibility of progression to a higher immunoarchitectural pattern or higher grade lymphoma, like DLBCL. (24) Although earlier reports suggested that nearly one half of the cases of DLBCL following NLPHL are of the THRLBCL type (31), but the current literature states that NLPHL can progress to DLBCL (NOS). (3) The clues towards progression to a higher grade lymphoma are a higher immunoarchitectural pattern and loss of FDC meshwork. Also there has found to be a clonal relationship between NLPHL and DLBL, but exact features that would predict this has not yet been established. (32) Hence long term follow up is important in this group of patients. (32)(33)(34)

GENETIC BASIS OF NLPHL:

Recent studies have shown that 50% of NLPHL cases are associated with BCL6 gene rearrangements, with a large number of them involving the IGH gene at 14q32. The other genetic rearrangements found in NLPHL include translocations involving 2q23, 5q31, 6q22, 9q22 and 17p21, chromosome 1q gain and losses of chromosome 4, 7 and 13. (35)

Gene expression profiling studies of micro dissected LP cells were done and have shown them to be characterised by the constitutive activity of nuclear factor κB. They were also found to be associated with aberrant extracellular signal regulated kinase signalling. Studies done by Brune et al., and Hartmann et al.,(36)(37) have shown NLPHL to be closely related to CHL and TCHRLBCL. The molecular differences between CHL and NLPHL are summarised in Table.3.

Table.3: Molecular differences between NLPHL and CHL

NLPHL	**CHL**
NFKBIA and TNFAIP3 mutations are absent	NFKBIA, NFKBIE and TNFAIP3 mutations present
Genomic gains in CREL absent	Genomic gains in CREL present
ERK pathway dysregulation is seen in a subset of cases	
Down regulation of signalling factors including CD79a, CD22, SYK and transcription factors including BOB1, E2A, PAX5 and Ikaros	

The differences between NLPHL and THRLBCL have been discussed elsewhere.

DIFFERENTIAL DIAGNOSIS OF NLPHL:

NLPHL as with any other disease has to be differentiated from its close counterparts which include: PTGC, lymphocyte rich classic Hodgkin lymphoma (LRCHL) and THRLBCL.

The features distinguishing PTGC from NLPHL has already been described above.

LRCHL and THRLBCL have been grouped together under the term ‘grey zone lymphomas’.

The REAL and WHO 2008 introduced the term borderline or grey zone lymphomas after much debate had been made on the lymphomas with overlapping features. (11)(3) These grey zone lymphomas typically had features of both Hodgkin as well as non Hodgkin lymphoma, and hence making a diagnosis was difficult. The role of immunohistochemistry has been found to be indispensible in these cases. Whether these diseases have a biological overlap or represent either ends of a spectrum of the same disease is still not fully understood.

The grey zone lymphomas include:

1. Nodular sclerosis classic Hodgkin lymphoma and primary mediastinal large B cell lymphoma.
2. Lymphocyte rich classic Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma.
3. Nodular lymphocyte predominant Hodgkin lymphoma and T-cell/histiocyte rich B-cell lymphoma. (38)

Since our study is limited to NLPHL and THRLBCL, we will limit our discussion to these two lymphomas and LRCHL.

Classic Hodgkin lymphoma is defined as a monoclonal lymphoid neoplasm composed of mononuclear Hodgkin cells and multinucleated RS cells in a polymorphous non-neoplastic background of small lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, fibroblasts and collagen fibres. (3)

Classic Hodgkin lymphoma is classified into 4 histologic subtypes, based on the morphology of the neoplastic HRS cells and the polymorphous background:

a) Lymphocyte rich classic Hodgkin lymphoma and nodular lymphocyte (LRCHL)
b) Nodular sclerosis classic Hodgkin lymphoma (NSCHL)
c) Mixed cellularity classic Hodgkin lymphoma (MCCHL)
d) Lymphocyte depleted classic Hodgkin lymphoma (LDCHL)

Morphologically, LRCHL is characterised by a nodular or diffuse pattern with the non-neoplastic background composed predominantly of lymphocytes and lacking neutrophils and eosinophils. The nodular pattern can often be confused with NLPHL, but one feature of LRCHL which distinguishes it from the latter is the presence of small or regressed germinal centres, usually at the periphery of the node. The HRS cells may resemble LP cells and are located within the nodules and outside the germinal centres. Occasionally NLPHL may have small germinal centres within the nodules. (2)(38)

Immunophenotyping is indispensible in the differentiation of LRCHL from NLPHL. Table.4 compares the immunoprofile of NLPHL and LRCHL.

On immunohistochemistry, the tumour cells of LRCHL are positive for CD30 and IRF4/MUM1, CD15+/-, CD20-/+, J chain -. The small lymphocytes within the nodules represent an expanded mantle zone and are positive for mantle zone markers IgM and D. Follicular dendritic cells are present which are CD21positive.

Table.4: Comparison of the immunoprofile of NLPHL and LRCHL

IMMUNOMARKERS	**NLPHL**	**LRCHL**
CD30	+/-	+
CD15	-	+/-
CD20	+	-/+
J chain	+/-	-
IRF4/MUM1	-	+

Hence with the above immunohistochemical markers, a differentiation between NLPHL and LRCHL can be made.

T CELL/HISTIOCYTE RICH LARGE B-CELL LYMPHOMA:

THRLBCL is a subtype of DLBCL.(39) The WHO 2008 defines diffuse large B-cell lymphoma as a neoplasm of the large B lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte that has a diffuse growth pattern. Based on the morphological, clinical, genetic and immunophenotypical DLBCL has been subdivided into many distinct entities / subgroups, of which TCRBCL is one of them. (40)

HISTORICAL BACKGROUND:

Ramsay et al., in 1988, described 5 cases of B-cell lymphoma with the histological and immunological features mimicking a T-cell lymphoma. These cases were previously diagnosed as peripheral T-cell lymphoma, based on the T-cell rich background. They described this lymphoma as T-cell rich B-cell lymphoma. (41)

Later in 1992, Delabie et al., reported 6 cases of B-cell lymphoma with a mixed nodular and diffuse infiltrate of reactive lymphocytes with a prominent histiocyte population which was obscuring the neoplastic large B-cells. The clonal nature of the B-cells was proven by immunoglobulin gene rearrangements in three of the six cases. All these cases were found to have a marked male preponderance, present at a late stage and were clinically aggressive. Two of these cases progressed to a diffuse large B-cell lymphoma. Based on the clinical features, morphology, immunohistochemical findings and clonality assays, they concluded that these cases represent a distinct subtype of B-cell lymphomas and termed them *'histiocyte rich B-cell lymphoma'.*

The above findings had not been verified by newer studies and the WHO 2001, recognised T-cell histiocyte rich lymphoma as a morphologic variant of DLBCL. (1)

In the following years, the trend shifted toward analysing the biology and molecular events of THRLBCL and analysing the relationship between this and NLPHL. The *Fifth International Congress on Hodgkin Lymphoma* (31) held a workshop to analyse the above features and also to set up diagnostic criteria and understand the therapeutic implications of grey zone lymphomas, of which THRLBCL and NLPHL are two among them. At the end of the workshop, precise diagnostic criteria could not be laid down and they suggested that THRLBCL is not a homogenous disease, with variations in morphology and immunophenotype.

In 2008, the WHO classified THRLBCL as a distinct subtype of DLBCL and they reconfirmed the suggestion that it is a heterogeneous disease. The neoplastic cells in THRLBCL may mimic the lymphocyte predominant cells of NLPHL, centroblasts, RS cells or Hodgkin cells.(3)

DEFINITION:

THRLBCL is defined by the WHO 2008, as one with limited number of scattered large atypical B-cells, embedded in a background of abundant T-cells and frequently histiocytes. These histiocytes are one of the distinct features used to make a diagnosis.

EPIDEMIOLOGY:

It is a very aggressive lymphoma and constitutes <10% of all DLBCL. It usually affects middle aged individuals in the 3rd or 4th decade (42)(43). This disease has a

male preponderance similar to that of NLPHL, with the male to female ratio being 2.6:1 (42).

SITES OF INVOLVEMENT:

The most common site of involvement is the lymph nodes, but extranodal disease, including liver, spleen and bone marrow is not uncommon.(42)

Other rare sites of involvement include skin(44), trigeminal ganglion(45), orbit(46), hard palate(47) and thyroid(48).

CLINICAL FEATURES:

The presenting symptoms of a patient with THRLBCL are fever, malaise and hepatospelonomegaly. They usually present at an advanced stage of disease with an intermediate to high International Prognostic Index (IPI) score.

MORPHOLOGY:

THRLBCL is characterised by a complete effacement effacement of architecture of the lymph node with a predominantly diffuse and occasionally vague nodular pattern with large B-cells set in a reactive background.

The neoplastic large B-cells may mimic the lymphocyte predominant cells of NLPHL, centroblasts, RS cells or Hodgkin cells. Wang et al., analysed 30 cases and attempted to characterise the origin of the neoplastic cells and found that 37% of their cases had more than one neoplastic cell morphology predominating in any given tumour. The postulated normal counterpart of the neoplastic B-cells is the germinal centre B-cell in some cases and heterogenous origins in other cases.(49) The WHO 2008 recommends

that cases with B-cells with variable sizes, morphology and distribution should not be classified as THRLBCL, instead grouped under DLBCL, NOS.(3)

The reactive background within which the neoplastic B-cells reside includes sheets of T-lymphocytes and histiocytes. The distinctive feature of this lymphoma is the presence of clusters of non epithelioid histiocytes. Other inflammatory cells such as plasma cells and eosinophils are not seen.

Reactive uninvolved B-lymphoid follicles with hypoplastic or hyperplastic germinal centres may be seen in the lymph node, usually at the periphery. Extracapsular invasion has been reported in many cases. Prominent blood vessels, fibrosis in the form of sclerotic bands and occasionally necrosis may be seen. (50)

Achten et al., observed that an increase in the number of neoplastic B-cells was associated with an increased susceptibility to progress to DLBCL. In their analysis of 40 patients, during the follow up, five patients were found to have a substantial increase in the number of large B-cells, and all 5 died due to the disease after it had progressed to DLBCL which was confirmed on autopsy. (42)

IMMUNOHISTOCHEMISTRY:

Due to the close resemblance to NLPHL and peripheral T cell lymphoma, immunohistochemical markers are required to confirm a diagnosis of THRLBCL.

The *neoplastic large B-cells* are positive for pan B-cell markers which include CD20, BCL6 in all cases and BCL2 and EMA in a small subset of cases.

These cells lack expression of CD15, CD30 and CD138.

The *small T-lymphocytes* are universally positive for T-cell markers which include CD3 and CD5.

The number of T-cells which are CD57 positive, range from very occasional to a significant number.

IgD(mantle cells) and follicular dendritic cell markers i.e., CD21 and CD23 are negative.

The *histiocytes* are CD68 positive.

COMPARISON BETWEEN NLPHL AND THRLBCL:

MORPHOLOGY (Table.5):

Table.5: Comparison of epidemiological and morphological features of NLPHL and THRLBCL

NLPHL	THRLBCL
Children and adolescents with a second peak between 30-50 years	3rd or 4th decade
Male preponderance	Male preponderance
Neoplastic lymphocyte predominant (LP cells) or popcorn cells	Neoplastic large B-cells may mimic the LP cells of NLPHL, centroblasts, RS cells or Hodgkin cells
6 variant immunoarchitectural patterns	No described patterns
The background is composed of FDC meshwork admixed with lymphocytes (predominantly B cells), epithelioid histiocytes and plasma cells	The background is composed of sheets of T cells and histiocytes

IMMUNOHISTOCHEMISTRY:

Table.6: Comparison of immunohistochemical features of NLPHL and THRLBCL

NLPHL	TCRLBCL
CD30-	CD30-
CD15-	CD15-
CD45+	CD45+
CD20+	CD20+
CD79a+	CD79a+
BSAP+	BSAP +
J chain+/-	J chain +/-
OCT2 S+	OCT2 S+
BOB1 +	BOB1 +
BCl 6 ++	BCl 6 -/+
CD21+	CD21-

As is evident from the above table, NLPHL and TCRLBCL have similar immune profiles with occasional differences.

There has been and continues to be immense work in the differentiation of NLPHL from THRLBCL, especially the diffuse variant. The importance of differentiating these two lymphomas is that they have a completely different prognosis and treatment.

Jaffe et al., in their effort to study the relationship between these two lymphomas, found that NLPHL and THRLBCL can occur in sequential and concurrent biopsies

from the same patients and can occur within families, thereby supporting a relationship between the two. (31)

De Jong et al., found that nearly one half of the cases of DLBCL following NLPHL are of the THRLBCL type. They suggested that the difference in the composition of the background cells may be due to the cytokines produced by the accessory cells during evolution to a higher grade. (31)

Rudiger et al., stated that THRLBCL may exhibit a nodular pattern. However, it is the small reactive B lymphocytes that are the major distinguishing feature between NLPHL and THRLBCL.(31)

Delabie et al., in their study of 10 cases of composite NLPHL and THRLBCL, found that there is reduced expression of transcription factor, PU.1, when NLPHL progresses to THRLBCL and whether this factor contributes to the progression was not known.(31)

Hartmann et al., used novel immunohistochemical markers including BAG6/BAT3, HIGD1A, UBD/FAT10 and CXCL13 on NLPHL, Pattern E NLPHL and THRLBCL.

They found that HIGD1A expression was stronger in NLPHL than THRLBCL, BAT/BAG6 expression was stronger in THRLBCL than NLPHL, UBD/FAT10 is more frequently expressed in Pattern E NLPHL and THRLBCL and only occasionally expressed in typical NLPHL. CXCL13 expression was seen in a few cases of variant NLPHL (including pattern C and E) and THRLBCL, in keeping with the fact that there is weak upregulation of CXCL13 in the neoplastic cells when compared with the germinal centre B cells.

Since the above mentioned novel immunohistochemical markers did not show any promising results, they tried to analyse the importance of the microenvironment in these lymphomas. Their findings were as follows:

1. CD4 T cell count was markedly lower in THRLBCL when compared to Pattern A NLPHL, but the difference between Pattern A and Pattern E NLPHL was not significant.
2. There was no significant difference in the CD8 T cell count between THRLBCL, Pattern A and Pattern E NLPHL.
3. The CD163 histiocyte count was significantly higher in Pattern E NLPHL and THRLBCL when compared to Pattern A and Pattern C NLPHL.
4. The percentage of T follicular helper cells was higher in Pattern A and C NLPHL when compared to Pattern E NLPHL and THRLBCL. (36)

PD1 AND CD57:

PD1:

PD1, also known as CD279, is a co-inhibitory receptor of lymphocytes and controls lymphocyte activation by providing negative signals in conjunction with signals from lymphocyte antigen receptors. It is expressed by germinal center-associated helper T cells; inhibits T cells (51) and is expressed by CD8+ T cells, associated with CD8 activation(52). It has two known ligands: PD-L1 (B7H1; CD274) and PD-L2 (B7DC; CD273). PD-1+ rosettes of T cells around neoplastic cells is relatively specific for nodular lymphocyte-predominant Hodgkin lymphoma. (51)

CD57:

CD57, also known as Leu7, beta-1,3-glucuronyltransferase 1 and glucuronosyl transferase P, is a glycoprotein with cell adhesion functions. It is expressed in 57% of NLPHL cases.

There have been two studies till date that have assessed the usefulness of PD1 and CD57 rosettes in NLPHL and THRLBCL. (4)(5)

Nam Cha et al., in 2008 analysed the efficacy of PD1, CD57 and other immunomarkers in NLPHL and its differential diagnosis. PD1 rosettes were seen in 98.3% NLPHL cases which were in contrast to CD57 which was seen in only 75.9% NLPHL cases. PD1 was found to be more sensitive than other markers, including CD57. In NLPHL with diffuse areas, PD1 expression was seen in 71.4% cases and limited to the nodular areas, whereas CD57 expression was seen in 100% cases and limited to the nodular areas. They also observed that the intensity of staining gradually reduced from the nodular to diffuse areas. In cases which were intermediate between NLPHL and THRLBCL, which included 5 cases, PD1 rosettes were seen in 80% cases in contrast to CD57 which was seen in 60% cases. None of the THRLBCL cases demonstrated PD1 rosettes. Thus they concluded that PD1 is a more sensitive marker and suggested it to be used as a routine immunomarker in NLPHL. (4)

Churchill et al., in 2010 compared PD1 and CD57 in the 6 patterns of NLPHL, de novo THRLBCL and nodular LRCHL. Their results were in keeping with Nam Cha et al's findings that PD1 is more superior to CD57 in the nodular variants of NLPHL-A,

B and D. (PD1 rosettes were seen in 87% of nodular NLPHL vs C57 rosettes seen in 50% of nodular NLPHL). Also the PD1 reactivity in Pattern C was present in 66% of cases when compared to 33% of cases with CD57. Another finding in their study was that there was gradual loss of expression of PD 1 from the nodular (56%) to diffuse (19%) areas. (5)

Since PD1 rosettes were present in THRLBCL cases also, they mentioned that the loss of PD1 does not correlate with progression to DLBCL/THRLBCL. (5)

GENE EXPRESSION PROFILING STUDIES:

De Wolf-Peeters et al., conducted a comparative genomic hybridisation on the micro dissected neoplastic cells of NLPHL and THRLBCL and found that NLPHL had more number of genetic imbalances (221 with an average of 11.6 per tumour) when compared to THRLBCL (91 imbalances with an average of 5.6 per tumour). They found several recurrent aberrations in the two with only a few overlapping in both the lymphomas. Thus they concluded that there may be a possible common origin for paragranuloma and THRLBCL, however, THRLBCL evolving from paragranuloma is less likely. (31)

Hartmann et al., in their effort to study the relationship or rather progression of NLPHL to THRLBCL, did a gene expression profile of micro dissected tumour cells of NLPHL, THRLBCL and pattern E NLPHL. (36)

On applying a false discovery rate (FDR) of 0.3, they found only some differentially expressed genes. This was done after a failed attempt on applying an FDR of 0.1. HIGD1A (hypoxia inducible domain family member 1A) was the sole differentially expressed gene in Pattern E NLPHL, which was 2.4 fold up regulated, in comparison with THRLBCL (p value <0.0001, FDR=0.104). On comparing typical NLPHL and THRLBCL, they found 16 genes which were up regulated 1.7 fold in typical NLPHL. These included HIGD1A, SEPT14 (GTP binding cytoskeletal protein), RGS13 (regulator of G protein signalling), AMY2A (amylase alpha 2A), RPS27 and MRPL51 (ribosomal proteins) and SNORD75 (small nucleolar RNA). 8 genes were found to be up regulated in THRLBCL, which included BAT3/BAG6 (HLA associated transcript) being the most upregulated (3.5 fold), MT2A (metallothionein 2A), MT1H (metallothionein 1H), CXCL9 (chemokine ligand 9) and S100A8 (S100 calcium binding protein A8).

On comparison with germinal centre B cells, 44 genes, 40 genes and 28 genes were up regulated in the neoplastic cells of typical NLPHL, Pattern E NLPHL and THRLBCL. A notable finding in their study was that UBD/FAT10, which modifies p53 and mediates NF-κB activation, was particularly up regulated.

They concluded that there is a molecular overlap between NLPHL and THRLBCL with no clear differences in the gene expression patterns of the neoplastic cells of NLPHL, THRLBCL and pattern E NLPHL. They also concluded that possibly Pattern E NLPHL and THRLBCL represent more aggressive forms of NLPHL and are most probably the same disease. (36)

Franke et al., in 2002 analysed 17 cases of THRLBCL, and attempted to identify the molecular basis of THRLBCL. They used a comparative genomic hybridisation along with microdissection of the neoplastic cells and DNA amplification by a polymerase chain reaction (DOP-PCR-CGH approach). The most commonly found genetic imbalances in THRLBCL and NLPHL were gain of Xq, Xp, 4q and 18q and loss of 17p and 19/19p. The genes encoded in these regions include BCL2 (18q21), MLT (18q21), and p53 (17p13), but their definite role in the pathogenesis of THRLBCL has not yet been fully analysed. Another interesting finding observed by Franke et al., was that the gain of 4q, which is a rare event in non Hodgkin lymphomas, was found to be high in both THRLBCL(41%) and NLPHL(~50%).

They also found significant differences between the two above mentioned lymphomas (Table.7).

Table.7: Molecular differences between NLPHL and THRLBCL according to Franke et al.

NLPHL	**THRLBCL**
High average number of chromosomal gains and losses	Less complex pattern of genomic imbalances
6-22 imbalances per tumour	1-5 imbalances per tumour

Hence they concluded that THRLBCL and NLPHL are two distinct lymphomas rather than two ends of a spectrum of the same disease. (53)

MATERIALS AND METHODS

MATERIALS AND METHODS

DATA COLLECTION:

Cases diagnosed as NLPHL and THRLBCL on lymph node biopsies between January 2003 and December 2013 in the Department of General Pathology, Christian Medical College and Hospital, Vellore were retrieved by using keyword search from the Oracle based pathology database. Cases for which archival slides and paraffin blocks were available were included in the study.

NLPHL:

The H&E and IHC stained slides of 52 biopsies of 50 patients and the blocks of 45 biopsies were retrievable from the Department of Pathology archives. Of the 45 blocks retrieved, only 34 blocks had sufficient tissue to run all immunohistochemical markers.

THRLBCL:

The H&E and IHC stained slides of 13 cases and the blocks of only 10 cases were retrievable from the Department of Pathology archives. Immunohistochemical markers were run on all of these 10 cases.

All 52 biopsies of NLPHL and 13 cases of THRLBCL were reviewed by two pathologists.

INCLUSION CRITERIA:

- All cases diagnosed as NLPHL or THRLBCL between January 2003 and December 2013 with diagnosis confirmed on review and for which archival material was available.
- Excision and core biopsies of lymph nodes.

EXCLUSION CRITERIA:

- Cases with alternative diagnosis on review.
- Insufficient tissue sample in the blocks for performing immunohistochemical markers.

CLINICAL DETAILS:

The clinical details of the cases were obtained from the Pathology workstation, Department of General Pathology. The clinical features analysed included age, gender, lymph node group involved and duration of symptoms.

HISTOPATHOLOGICAL ASSESSMENT:

The gross and microscopic features of NLPHL and THRLBCL were assessed as follows:

GROSS FEATURES:

1) Lymph node size: The gross size of the lymph node in excision specimens was assessed.

2) Cut surface: The colour and consistency of the lymph nodes involved were assessed.

MICROSCOPIC FEATURES:

1) Capsule: The presence or absence of capsule was assessed. If present, the thickness of the capsule was assessed.
2) Effacement of architecture: The architecture of the lymph node was assessed on scanner view (4X). The effacement whether partial or complete was assessed.
3) Nodularity: The percentage of nodularity in the lymph node was assessed on scanner view (4X).
4) Peri-nodal infiltration: The peri-nodal tissue was assessed for the presence or absence of infiltration by the neoplastic cells.
5) Back to back arrangement of nodules: The presence or absence of back to back arrangement of nodules was assessed.
6) Shape of nodules: The shape of the nodules was assessed whether they were round or serpiginous.
7) Diffuse areas: The presence or absence of diffuse areas was assessed. If present, the percentage of diffuse areas was assessed.
8) Small germinal centres: The presence or absence of small germinal centres within the lymph node was assessed.
9) Sclerosis: The presence or absence of sclerosis was assessed. If present, the extent of sclerosis, whether focal or extensive was assessed.

10) Granulomas: All lymph node biopsies were thoroughly searched for the presence of granulomas. If present, the slides of special stains for acid fast bacilli were assessed.

11) LP cells: The H&E stained sections were assessed for the presence or absence of LP cells. When present, the location of the LP cells, whether intranodular or extranodular was assessed.

12) RS like cells: The presence or absence of RS like cells was assessed in the H&E stained sections. When present, their location, whether intranodular or extranodular was noted. The presence or absence of perinucleolar halo and inclusion like nucleoli was noted.

13) Residual normal tissue with reactive follicles: The lymph nodes were assessed for the presence of residual normal tissue with reactive follicles.

14) Progressive transformation of germinal centres: The presence or absence of PTGC was noted.

15) Other cells: The lymph nodes were examined for the presence of inflammatory cells like histiocytes, plasma cells and eosinophils.

The additional microscopic features that were assessed in THRLBCL were:

1) Prominent blood vessels: The presence of prominent high endothelial venules within the lymph node was assessed.
2) Necrosis: The presence or absence of necrosis was assessed.

IMMUNOHISTOCHEMISTRY:

Two immunohistochemical markers were selected for our study, PD1 and CD57. These markers were performed on 34 biopsies only. The protocol followed for automated immunostaining of PD1 and CD57 is given in the Appendix.

The details of the antibodies used are summarised in Table.8.

Table.8: Antibody clones used for immunohistochemical markers PD1 and CD57

ANTIBODY	CLONE	DILUTION	SOURCE	TECHNIQUE
PD-1	MRQ-22	Predilute	Cellmarque	Ventana Benchmark XT autostainer
CD 57	NK-1	Predilute	Leica	Ventana Benchmark XT autostainer

The grading system used for PD1 and CD57 rosettes is as follows:

- Frequent: ≥6% PD1/CD57 positive rosettes
- Infrequent: 1%-5% PD1/CD57 positive rosettes
- Absent: no PD1/CD57 positive rosettes.(5)

1. PD1: Freshly cut and stained PD1 sections were assessed for the presence of T cell rosettes surrounding the neoplastic large B cells. The percentage of PD1 rosettes,

whether frequent, infrequent or absent was noted. Tonsillar tissue was used as an external control for all cases.

2. CD57: The CD57 stained sections were assessed for the presence of T cell rosettes surrounding the neoplastic large B cells. The percentage of CD57 rosettes, whether frequent, infrequent or absent was noted. Tonsillar tissue was used as an external control for all cases.

   Cases in which PD-1 and CD57 did not work were excluded from the analysis of immunohistochemical results.

   The following were assessed on the already stained sections of NLPHL. Re-staining was done in those cases where the stains had faded away.

3. CD15 and CD30: The LP cells were examined for staining by CD15 and CD30. When positive, the nature of staining, membrane or Golgi staining and the percentage of positive cells were assessed.

4. CD20: The CD20 stained sections were examined and the percentage of these small B cells within and outside the nodules was noted separately.

   Similarly, the percentage of large B cells within and outside the nodules was noted separately.

5. CD3: The CD3 stained sections were examined and the percentage of T lymphocytes within the nodules and outside the nodules was noted separately. The proportion of T

& B lymphocytes in the intranodular small lymphocytic population and the extranodular small lymphocytic population were also noted separately.

6. Other IHC markers: The biopsies which had other IHC stained slides were reviewed. The IHC markers which were examined in our cases included: OCT 2, BOB 1, MIB-1, LCA, PAX-5, MUM-1, BCl-6, CD79a, EMA, EBV-LMP, Alk-1, CD4, CD8, CD56 and Granzyme B.

CHARACTERISATION INTO VARIANT IMMUNOARCHITECTURAL PATTERNS:

The NLPHL biopsies were classified into the variant immunoarchitectural patterns as described by Fan et al (2) (see Appendix.1) as follows:

A) "Classic" nodular pattern, B cell rich
B) Serpiginous/interconnected nodular pattern
C) Nodular with prominent extranodular L&H cells
D) Nodular with T- cell-rich background
E) Diffuse pattern (T-Cell-Rich B-Cell Lymphoma-like)
F) (Diffuse), "Moth-eaten" with B-cell-rich background

If more than one pattern was present in the lymph node, the percentage of the major and minor patterns was noted.

STATISTICAL ANALYSIS:

The study data was summarised using Epi Info software, Version7.1.3.3. All statistical analysis was performed using SPSS software, Version Stata/IC13.1. Demographics and categorical variables were summarised using descriptive analysis. The usefulness of the two markers PD1 and CD57 in diagnosing NLPHL and THRLBCL were assessed by calculating their sensitivity, specificity, positive and negative predictive values and positive likelihood ratio using 2x2 tables.

RESULTS

RESULTS

Cases diagnosed as NLPHL and THRLBCL on lymph node biopsies between January 2003 and December 2013 in the Department of General Pathology, Christian Medical College and Hospital, Vellore were retrieved by using a keyword search from the Oracle based pathology database. Cases for which archival slides and paraffin blocks were available were included in the study.

NLPHL:

The H&E and IHC stained slides of 52 biopsies of 49 patients diagnosed as NLPHL and the blocks of 45 biopsies were retrievable from the Department of Pathology archives. Of the 45 blocks retrieved, only 34 blocks had sufficient tissue to run all immunohistochemical markers.

Two patients had a relapse each after two years of diagnosis and another patient had 2 biopsies, one of which was a block review from elsewhere.

THRLBCL:

The H&E and IHC stained slides of 13 cases and the blocks of only 10 cases were retrievable from the Department of Pathology archives. Immunohistochemical markers were run on all of these 10 cases.

CLINICAL FEATURES:

AGE:

The mean age at diagnosis of NLPHL was found to be 31 years, with a standard deviation of 14.181 (Table.9). The youngest patient was 9 years and the oldest patient was 73 years old. Our study included 12 cases which were in the paediatric age group (<18 years of age). Fig.1 shows the distribution of cases according to age in our study.

Table.9: Measures of central tendency for age in NLPHL cases included in our study

Total number of cases	**Mean**	**Standard deviation**	**Median**
52	31.42	14.18	31

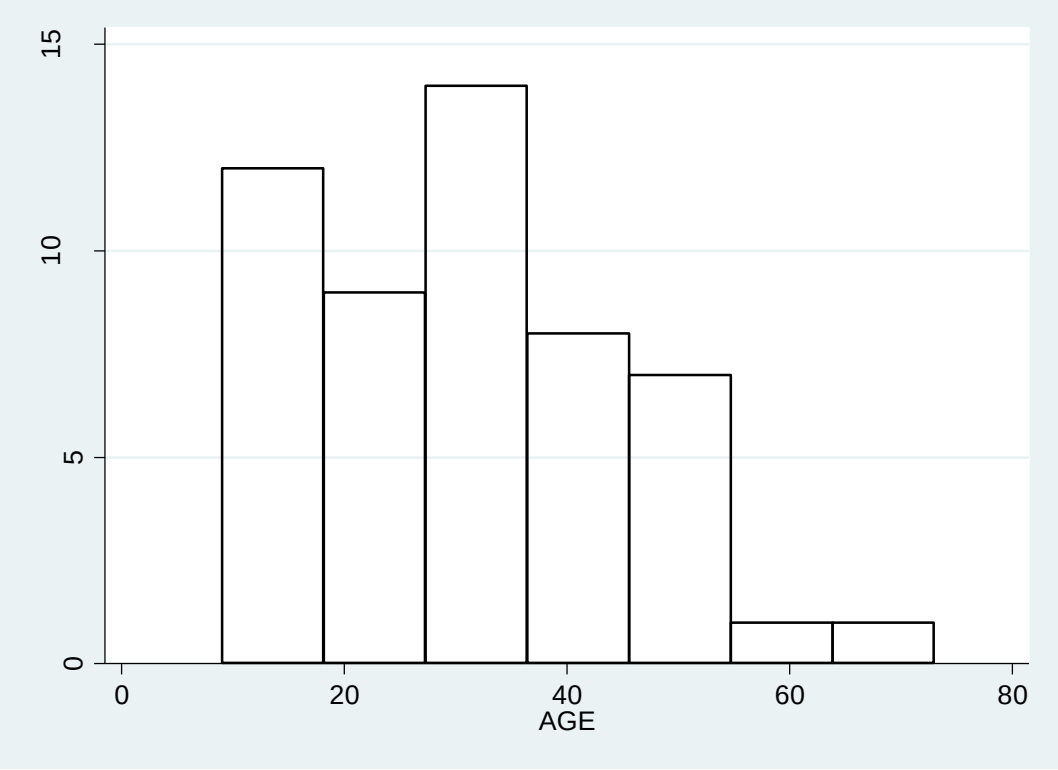


Figure.1: Age at diagnosis of NLPHL

GENDER:

NLPHL showed a marked male preponderance with 85.7% (42 of 49 cases) of the patients being male and only 14.3% (7 of 49 cases) being female (Fig.2).

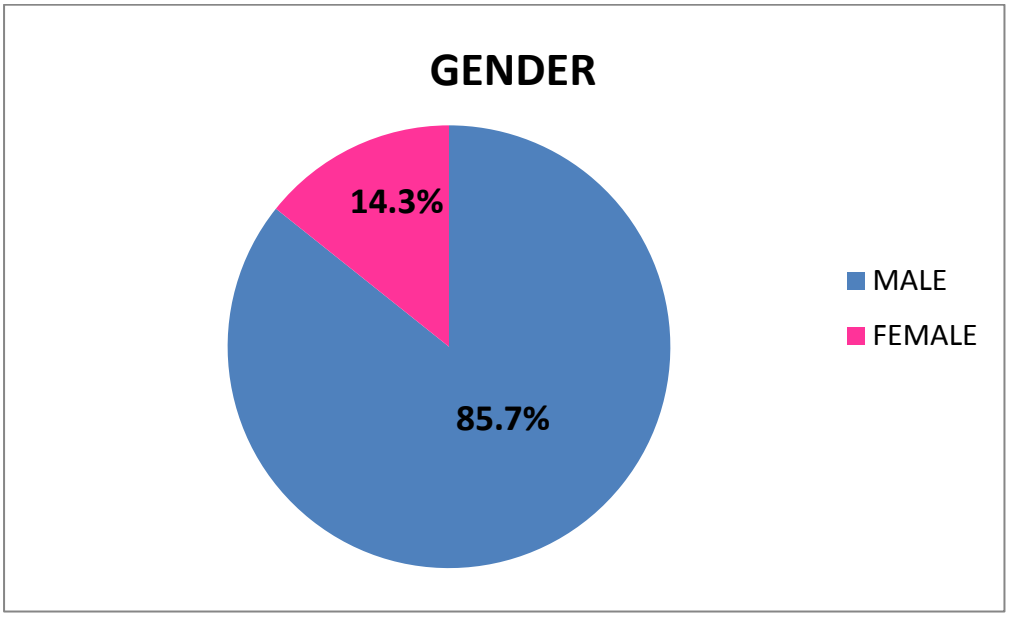


Figure.2: Gender distribution of NLPHL

LYMPH NODE GROUP INVOLVED:

The most common lymph node group involved was found to be the cervical lymph nodes, followed by axillary and inguinal lymph nodes. The other less commonly involved lymph node groups observed in our study were pre-auricular, supra-clavicular, intra-abdominal and para-aortic lymph nodes in order of their frequencies. One patient had generalised lymphadenopathy. (Table.10) The exact location of lymph node involvement was not known in 2 cases. Five of the patients (10.2%) included in our study had definite bone marrow involvement.

Table.10: Lymph node groups involved

LYMPH NODE GROUP	NO. OF BIOPSIES
AXILL	10
CERV	18
ING	11
AXILL/ING	2
AXILL/SUPRACLAV	1
AXILL/ING/CERV	1
AXILL/ING/SUPRACLAV	1
INTRA-ABDOMINAL	1
PRE-AURICULAR	3
GENERALISED	1
PARA-AORTIC	1
TOTAL	**50**

LYMPH NODE GROUP INVOLVEMENT IN CHILDREN AND ADOLESCENTS:

Our study included 12 biopsies which were in the paediatric age group (<18 years of age) and the frequency of involvement of each lymph node group is as follows:

Cervical 33.4% (4 out of 12 biopsies), inguinal 25% (3 out of 12 biopsies), axillary 16.7% (2 out of 12 biopsies), pre-auricular, intra abdominal and para-aortic 8.3% each (1 out of 12 biopsies each).

DURATION OF SYMPTOMS:

The duration of symptoms ranged from 1 month to 30 years.

CAPSULE:

Of the 52 biopsies included in our study, the capsule could be assessed in only 39 biopsies. Thickening of capsule was identified in 24 of 39 biopsies (61.5%) and the capsule was normal in the remaining 15 of 39 biopsies (38.5%).

EFFACEMENT OF ARCHITECTURE:

Effacement of architecture was assessed in 49 of 52 biopsies, as 3 of the biopsies were core biopsies. There was complete effacement in 46 of 49 biopsies (94%) and partial effacement in only 3 of 49 biopsies (6%).

NODULARITY:

Nodularity was assessed in 50 of 52 biopsies because of the 3 core biopsies; two of them did not show nodular areas. 2 of 50 biopsies (4%) had ≤10% and 11-50% nodularity each. The remaining 46 of 50 biopsies (92%) had >50% nodularity in the lymph nodes examined. (Table.11)

Table.11: Frequency and percentage of biopsies with ≤10%, 11-50% and >50% nodularity in NLPHL

NODULARITY	FREQUENCY	PERCENTAGE (%)
≤10%	2	4
11 - 50%	2	4
>50%	46	92
Total	**50**	**100**

PERINODAL INFILTRATION:

Perinodal infiltration was assessed in 47 biopsies, of which only 1 biopsy (2%) had perinodal infiltration and the remaining 46 of 47 biopsies (98%) did not have any evidence of the same.

BACK TO BACK ARRANGEMENT OF NODULES:

46 of 50 biopsies had back to back arrangement of nodules and the remaining 4 of 50 biopsies did not have the same. One of the 3 core biopsies had focal back to back arrangement of nodules and the same could not be assessed in the other 2 core biopsies.

SHAPE OF NODULES:

Of the 52 biopsies, nodules were present in 51 biopsies. 47 of 51 biopsies had round nodules only, 2 of 51 biopsies had serpiginous nodules only and 2 of 51 cases had both round and serpiginous nodules within the same lymph node.

DIFFUSE AREAS:

12 of 52 biopsies had diffuse areas and the percentage of diffuse areas ranged from 20% to 90% respectively. 8 of 12 biopsies (66.67%) had diffuse areas ranging from 11 to 50% within the nodes and 4 of 12 biopsies (33.33%) had >50% diffuse areas.

SMALL GERMINAL CENTERS:

Only 1 of 52 biopsies had small germinal centres within the nodules.

SCLEROSIS:

Sclerosis was assessed in all 52 biopsies and was found in 32 of 52 biopsies (61.5%). 30 of these 32 biopsies (94%) had only focal sclerosis and the remaining 2 of 32 biopsies (6%) had extensive sclerosis. (Fig.2)

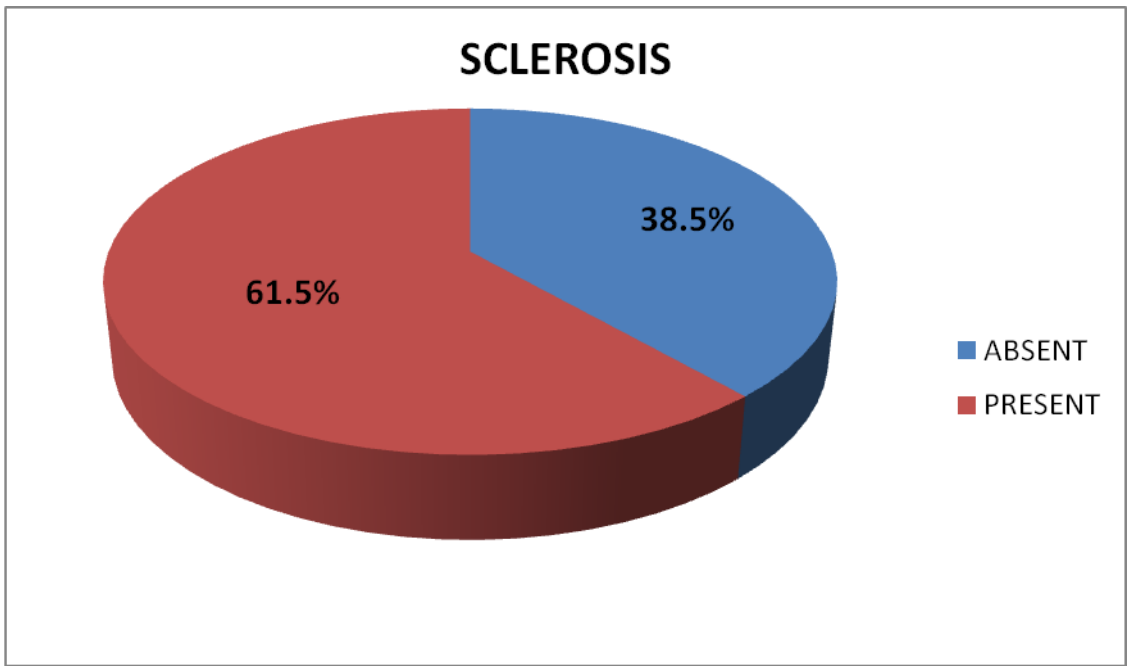


Figure.3: Sclerosis in NLPHL

GRANULOMAS:

Of the 52 biopsies of NLPHL included in our study, 5 biopsies (10%) had granulomas in the lymph node examined.

LP CELLS:

In our study, we assessed the site of the LP cells on H& E sections as well as CD20 stained sections, whether within or outside the nodules. Of the 52 biopsies, 49 biopsies (94%) had LP cells within the nodules and only 3 biopsies (6%) did not have any intranodular LP cells. (Table.12)

15 of 52 biopsies (29%) had extranodular LP cells and 37 of 52 biopsies (71%) did not have extranodular LP cells. (Table.13)

Table.12: Percentage of biopsies with and without intranodular LP cells

	NO. OF BIOPSIES	**PERCENTAGE (%)**
Absent	3	6
Present	49	94

Table.13: Percentage of biopsies with and without extranodular LP cells

	NO. OF BIOPSIES	**PERCENTAGE (%)**
Absent	37	71
Present	15	29

RS LIKE CELLS:

In our study, 30 of 52 biopsies (57.69%) had RS like cells exclusively within the nodules, 2 of 52 biopsies (3.85%) had RS like cells exclusively outside the nodules and 4 of 52 biopsies (7.69%) had RS like cells both within and outside the nodules. 16 biopsies (30.77%) did not have RS like cells in the sections examined. (Table.14)

Table.14: Distribution of RS like cells within the lymph node in NLPHL

RS LIKE CELLS	FREQUENCY	PERCENTAGE (%)
Absent	16	30.77
Within nodules only	30	57.69
Outside nodules only	2	3.85
Both within and outside nodules	4	7.69
Total	**52**	**100**

On assessing the type of RS like cells, 21 of 36 biopsies (58%) had only mononuclear RS like cells and 15 of 36 biopsies (42%) had both mononuclear and binuclear RS like cells.

RESIDUAL NORMAL TISSUE WITH REACTIVE FOLLICLES:

7 of 52 biopsies (13%) had associated residual normal tissue with reactive follicles in the lymph node biopsy examined.

PROGRESSIVE TRANSFORMATION OF GERMINAL CENTRES:

None of the 52 biopsies included in our study had progressive transformation of germinal centres in the lymph nodes examined.

OTHER CELLS:

The other cells which were assessed in our study include histiocytes, plasma cells and eosinophils. All 52 biopsies (100%) had histiocytes, 5 of 52 biopsies (10%) had rare plasma cells and 4 of 52 biopsies (8%) had rare eosinophils in the background.

PROGRESSION TO DLBCL:

Of the 49 cases included in our study, 3 cases (6.12%) had areas with progression to DLBCL (NOS). 2 of these 3 cases had a predominant pattern D and 1 case had pattern A in the NLPHL component examined.

RELAPSE/ RECURRENCE:

2 of 49 patients included in our study had a documented relapse. One patient initially presented with NLPHL-Pattern C and progressed to NLPHL-Pattern F after 2 years and the other patient progressed from NLPHL-Pattern A to NLPHL-Pattern D after 2 years.

IMMUNOHISTOCHEMISTRY:

VARIANT IMMUNOARCHITECTURAL PATTERNS:

In our study, we classified 51 biopsies of NLPHL into the 6 immunoarchitectural patterns as described by Fan et al. (2) (See Appendix.2) The pattern could not be assessed in one biopsy as it was a core biopsy and was insufficient to categorise into a specific pattern. Two other core biopsies had multiple cores and hence a pattern could be assigned to each of them.

The biopsies were classified into the variant patterns based on the assessment of the H&E, CD20 and CD3 stained sections. The overall frequency of each pattern in our study group is as follows (Fig.4):

Classic nodular pattern (A) was seen in 25 biopsies, with 24 of these biopsies having it as their major pattern (47.1%) and 1 biopsy having a minor component of Pattern A.

Serpiginous/interconnected nodular pattern (B) was seen in 4 biopsies, of which one case had it as a minor pattern and the remaining 3 cases had it as their major pattern (5.9%).

Nodular pattern with prominent extra nodular L&H cells (C) was seen in 2 biopsies (3.9%).

Nodular pattern with T cell rich background (D) was seen in 19 biopsies with 2 of 19 biopsies having it as a minor pattern. The remaining 17 biopsies had Pattern D as their major pattern (33.3%).

Diffuse pattern (T cell rich B cell lymphoma like) (E) was seen in 5 biopsies with 2 of 5 biopsies having it as the major pattern (3.9%) and the remaining 3 biopsies with it as their minor pattern.

Only 3 biopsies (5.9%) had a moth eaten pattern with B cell rich background (F).

One patient had two biopsies from different lymph nodes with one node showing Pattern A, the other showing Pattern B.

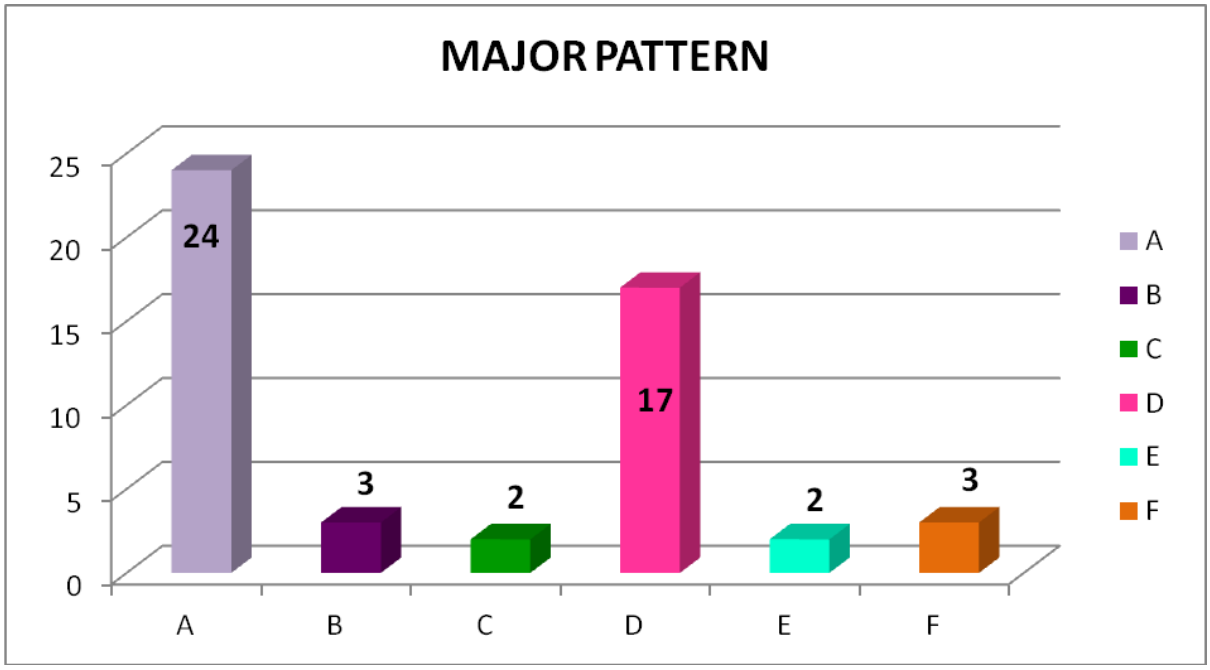


Figure.4: Frequency of the variant immunoarchitectural patterns of NLPHL in adults and children (x axis: Patterns, y axis: No. of cases)

Among the 12 paediatric biopsies included in our study, one was a core biopsy and was not assigned a pattern. The prevailing patterns observed in this group were Patterns D and A (5 cases each) and one case was Pattern E. (Fig.5)

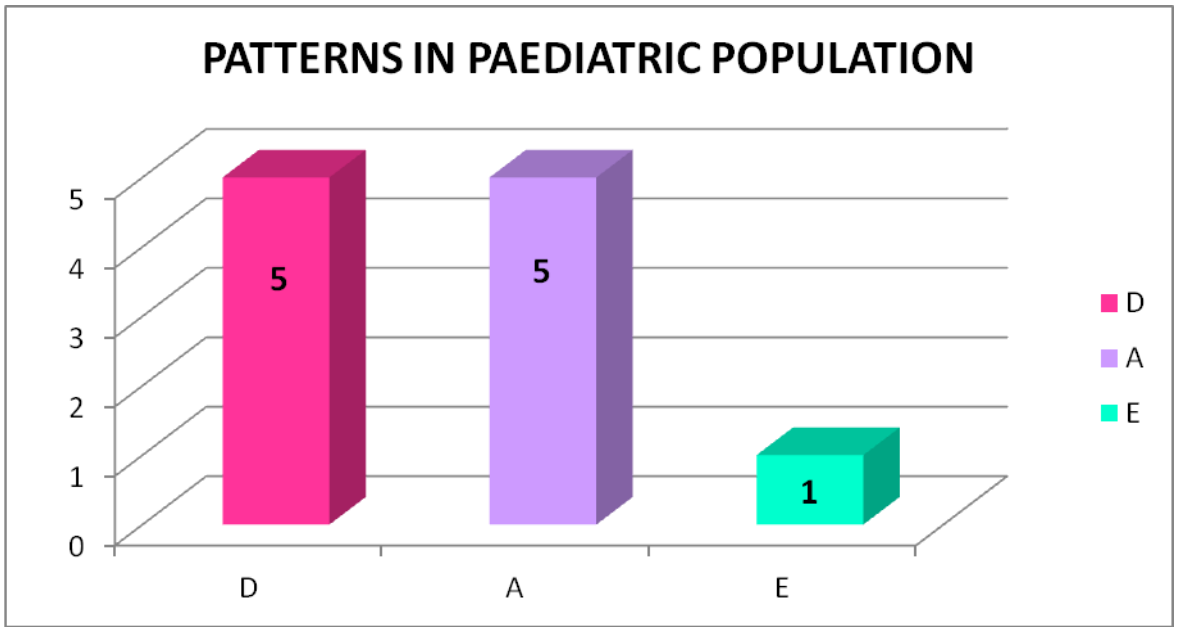


Figure.5: Frequency of the variant immunoarchitectural patterns of NLPHL in the paediatric population (x axis: Patterns, y axis: No. of cases)

Hybrid/mixed patterns were also observed in our study. Of the 51 biopsies included in our study, 44 biopsies had a pure/ single pattern and 7 biopsies had a mixture of two patterns. The hybrid/ mixed patterns observed were pattern D / E in 4 of 7 biopsies, pattern A / B in 2 of 7 biopsies and Pattern A / D in 1 biopsy. We found that Pattern E was always associated with Pattern D, suggesting that the T cell rich nodular pattern progresses to a diffuse pattern.

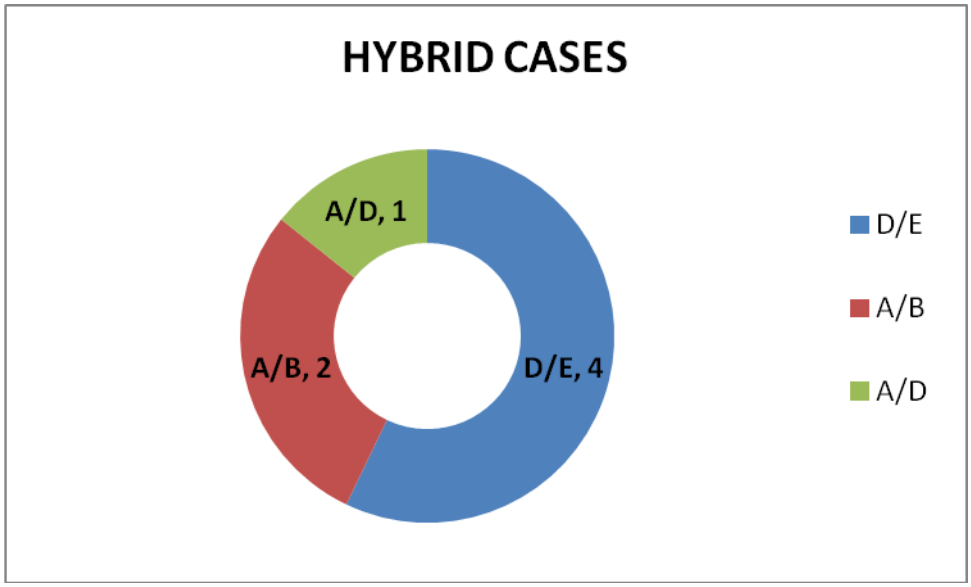


Figure.6: Hybrid patterns in NLPHL

ASSESSMENT OF SMALL AND LARGE B CELLS, INTRANODULAR AND EXTRANODULAR T AND B LYMPHOCYTES:

In our study, the percentage of small and large B cells within and outside the nodules was assessed in the CD20 stained sections. The T and B lymphocytes within and outside nodules were assessed in the CD3 and CD20 stained sections.

PATTERN A:

Among the cases classified as pattern A, which included 24 biopsies, the percentage of small B cells within nodules ranged from 70% to 95% with a mean of 83.54% and median of 80%. The percentage of small B cells outside nodules ranged from 5% to 30% with a mean of 16.46% and median of 20%.

The large B cells within nodules ranged from 10% to 100% with a mean of 88.54% and median of 92.50%. Only 15 of 24 biopsies (62.5%) had large B cells outside nodules which ranged from 5% to 90% with a mean of 18.33% and median of 10%.

The percentage of intranodular lymphocytes which were T ranged from 10% to 50% with a mean 27.92% and median of 30% and which were B ranged from 50% to 90% with a mean of 72.08% and median of 70%.

The percentage of extranodular lymphocytes which were T ranged from 60% to 95% with a mean 81.88% and median of 80% and which were B ranged from 5% to 40% with a mean of 18.33% and median of 20%. (Table.15)

Table.15: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern A

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	70	95	83.54	80	7.29
Outside nodules	5	30	16.46	20	7.29
LARGE B CELLS					
Within nodules	10	100	88.54	92.50	19.48
Outside nodules*	5	90	18.33	10	22.09
INTRANODULAR					
T lymphocytes	10	50	27.92	30	10.62
B lymphocytes	50	90	72.08	70	10.62
EXTRANODULAR					
T lymphocytes	60	95	81.88	80	9.42
B lymphocytes	5	40	18.33	20	9.42

* Present in only 15 of 24 cases

PATTERN B:

Among the 3 cases classified as pattern B, the percentage of small B cells within nodules ranged from 70% to 80% with a mean of 76.67% and median of 80%. The percentage of small B cells outside nodules ranged from 20% to 30% with a mean of 23.33% and median of 20%.

The large B cells within nodules ranged from 50% to 80% with a mean of 66.67% and median of 70%. The large B cells outside nodules ranged from 20% to 50% with a mean of 33.33% and median of 30%.

The percentage of intranodular lymphocytes which were T ranged from 20% to 40% with a mean 33.33 % and median of 40% and those which were B ranged from 60% to 80% with a mean of 66.67% and median of 60%.

The percentage of extranodular lymphocytes which were T ranged from 70% to 80% with a mean 76.67% and median of 80% and which were B ranged from 20% to 30% with a mean of 23.33% and median of 20%. (Table.16)

Table.16: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern B

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	70	80	76.67	80	5.77
Outside nodules	20	30	23.33	20	5.77
LARGE B CELLS					
Within nodules	50	80	66.67	70	15.28
Outside nodules	20	50	33.33	30	15.28
INTRANODULAR					
T lymphocytes	20	40	33.33	40	11.55
B lymphocytes	60	80	66.67	60	11.55
EXTRANODULAR					
T lymphocytes	70	80	76.67	80	5.77
B lymphocytes	20	30	23.33	20	5.77

PATTERN C:

2 cases were classified as pattern C.

The percentage of small B cells within nodules ranged from 80% to 90% with a mean and median of 85%. The percentage of small B cells outside nodules ranged from 10% to 20% with a mean and median of 15%.

The large B cells within nodules ranged from 20% to 40% with a mean and median of 30%. The large B cells outside nodules ranged from 60% to 80% with a mean and median of 70%.

The percentage of intranodular lymphocytes of the type T ranged from 20% to 30% with a mean and median of 25% and the percentage of intranodular lymphocytes of the type B ranged from 70% to 80% with a mean and median of 75%.

The percentage of extranodular lymphocytes which were T ranged from 70% to 80% with a mean and median of 75% and those which were B ranged from 20% to 30% with a mean and median of 25%. (Table.17)

Table.17: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern C

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	80	90	85	85	7.07
Outside nodules	10	20	15	15	7.07
LARGE B CELLS					
Within nodules	20	40	30	30	14.14
Outside nodules	60	80	70	70	14.14
INTRANODULAR					
T lymphocytes	20	30	25	25	7.07
B lymphocytes	70	80	75	75	7.07
EXTRANODULAR					
T lymphocytes	70	80	75	75	7.07
B lymphocytes	20	30	25	25	7.07

PATTERN D:

17 cases were classified as pattern D.

The percentage of small B cells within nodules ranged from 0% to 90% with a mean of 70.59% and median of 80%. The percentage of small B cells outside nodules ranged from 0% to 30% with a mean of 16.47% and median of 20%.

The large B cells within nodules ranged from 50% to 100% with a mean of 89.41% and median of 90%. Only 9 of 17 cases (52.94%) had large B cells outside nodules, which ranged from 10% to 50% with a mean and median of 20%.

The percentage of intranodular lymphocytes which were T ranged from 50% to 90% with a mean 74.71% and median of 80%. The percentage of intranodular lymphocytes which were B ranged from 10% to 50% with a mean of 25.29% and median of 20%.

Extranodular T lymphocytes were present in 16 of 17 cases (94.11%) and ranged from 60% to 100% with a mean 81.88% and median of 80%. Extranodular B lymphocytes were present in 15 cases and ranged from 10% to 40% with a mean of 19.33% and median of 20%. 1 case (Case no: 3) did not have extranodular T or B lymphocytes. In one other case (Case no: 20), 100% of the extranodular lymphocytes were T lymphocytes. (Table.18)

Table.18: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern D

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	0	90	70.59	80	28.39
Outside nodules	0	30	16.47	20	9.31
LARGE B CELLS					
Within nodules	50	100	89.41	90	13.91
Outside nodules*	10	50	20	20	13.23
INTRANODULAR					
T lymphocytes	50	90	74.71	80	11.25
B lymphocytes	10	50	25.29	20	11.25
EXTRANODULAR					
T lymphocytes**	60	100	81.88	80	10.47
B lymphocytes***	10	40	19.33	20	9.61

* Present in only 9 of 17 cases

** Present in 16 of 17 cases

*** Present in 15 of 17 cases

PATTERN E:

2 cases were classified as pattern E.

The percentage of small B cells within nodules ranged from 70% to 90% with a mean and median of 80%. The percentage of small B cells outside nodules ranged from 10% to 30% with a mean and median of 20%.

The large B cells within nodules ranged from 30% to 70% with a mean and median of 50%. The large B cells outside nodules ranged from 30% to 70% with a mean and median of 50%.

The intranodular and extranodular lymphocytes could be assessed in only 1 case. The other case had predominantly diffuse areas with majority of the cells being T lymphocytes, and hence the percentage of T and B lymphocytes within and outside the nodules could not be assessed.

The percentage of intranodular lymphocytes which were T was 80%, B was 20%, the percentage of extranodular lymphocytes which were T was 70% and B was 30% respectively. (Table.19)

Table.19: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern E

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	70	90	80	80	14.14
Outside nodules	10	30	20	20	14.14
LARGE B CELLS					
Within nodules	30	70	50	50	28.28
Outside nodules	30	70	50	50	28.28
INTRANODULAR					
T lymphocytes*	80	80	80	80	
B lymphocytes*	20	20	20	20	
EXTRANODULAR					
T lymphocytes*	70	70	70	70	
B lymphocytes*	30	30	30	30	

* Present in only 1 of 2 cases

PATTERN F:

3 cases were classified as pattern F.

The percentage of small B cells within nodules ranged from 20% to 80% with a mean of 60% and median of 80%. The percentage of small B cells outside nodules ranged from 20% to 80% with a mean of 40% and median of 20%.

The large B cells within nodules ranged from 20% to 90% with a mean of 46.67% and median of 30%. The large B cells outside nodules ranged from 10% to 80% with a mean of 53.33% and median of 70%.

The percentage of intranodular lymphocytes which were T ranged from 40% to 80% with a mean 63.33% and median of 70% and which were B ranged from 20% to 60% with a mean of 36.67% and median of 30%.

The percentage of extranodular lymphocytes which were T ranged from 20% to 70% with a mean 50% and median of 60% and which were B ranged from 30% to 80% with a mean of 50% and median of 40%. (Table.20)

Table.20: Measures of central tendency in percentage and standard deviation of T and B cells within and outside the nodules in Pattern F

	MINIMUM (%)	MAXIMUM (%)	MEAN (%)	MEDIAN (%)	STD DEVIATION
SMALL B CELLS					
Within nodules	20	80	60	80	34.64
Outside nodules	20	80	40	20	34.64
LARGE B CELLS					
Within nodules	20	90	46.67	30	37.86
Outside nodules	10	80	53.33	70	37.86
INTRANODULAR					
T lymphocytes	40	80	63.33	70	20.82
B lymphocytes	20	60	36.67	30	20.82
EXTRANODULAR					
T lymphocytes	20	70	50	60	26.46
B lymphocytes	30	80	50	40	26.46

CD15:

In our study, we assessed the previously stained CD 15 slides for positivity, and in those which were positive, the staining pattern was also assessed. Of 52 biopsies, 3 biopsies (6%) had very occasional CD15 positive cells with weak membrane staining. The percentage of cells which were CD15 positive was 3% in 2 of 3 biopsies and 5% in the other biopsy.

CD30:

16 of 52 biopsies (31%) had CD30 positive cells of which 12 biopsies (75%) had < 10% CD30 positive cells, 3 biopsies (19%) had 11-30% CD30 positive cells and 1 biopsy(6%) had ≥50% CD30 positive cells. All 16 biopsies had membrane staining and 2 of them had Golgi staining as well.

PD1 AND CD57 IN NLPHL:

In our study, which included 52 biopsies of NLPHL, immunohistochemical markers for PD1 and CD57 were run in 34 biopsies.

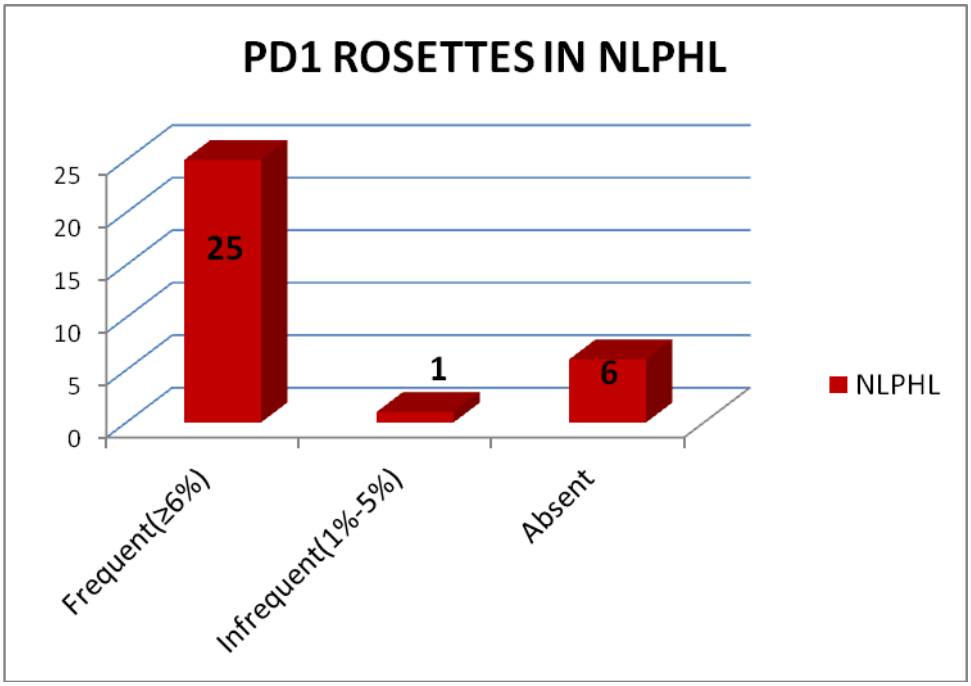
We found that the antigen preservation was better when the staining with PD1 antibody was done on freshly cut sections. However, the intensity of staining was weak.

25 of 32 biopsies (78.13%) showed frequent PD1 rosettes encircling the neoplastic large B cells, 1 of 32 biopsies (3.12%) showed infrequent PD1 rosettes and 6 of 32

biopsies (18.75%) did not have any PD1 rosettes in the presence of positive internal and external controls. PD1 did not work in 2 of the 34 biopsies. (Fig.7)

19 of 34 biopsies (55.9%) showed frequent CD57 rosettes, 7 of 34 biopsies (20.6%) showed infrequent CD57 rosettes and 8 of 34 biopsies (23.5%) did not have any CD57 rosettes in the presence of positive internal and external controls. (Fig.8)

Overall, PD1 staining was found to be more superior to CD57 in the diagnosis of NLPHL. All 6 biopsies which lacked PD1 rosettes also lacked CD57 rosettes.



* PD1 did not work in 2 of 34 cases

Figure.7: PD1 rosettes in NLPHL (x axis: Percentage of PD1 rosettes, y axis: No. of cases)

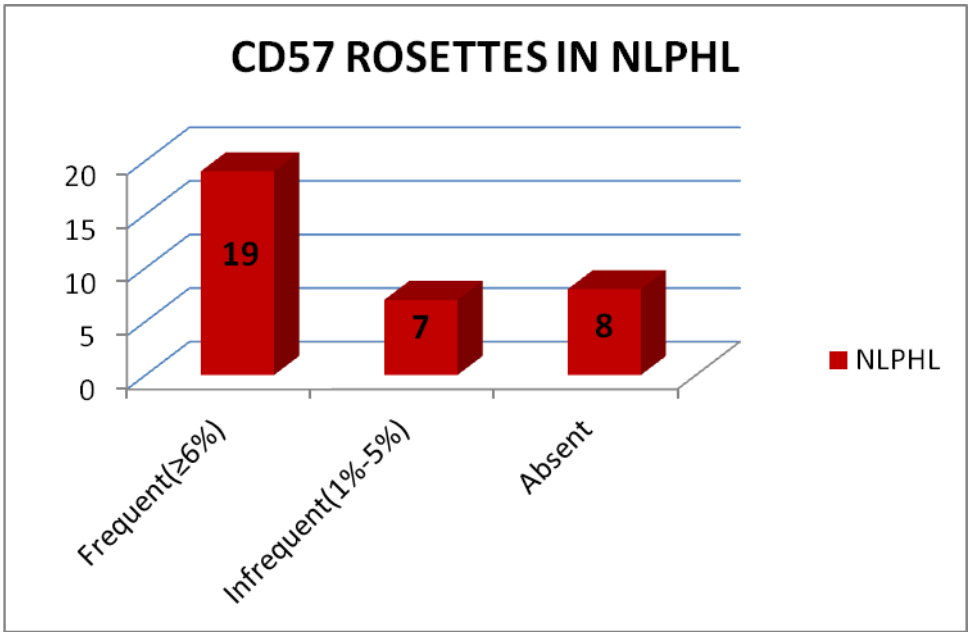


Figure.8: CD57 rosettes in NLPHL (x axis: Percentage of CD57 rosettes, y axis: No. of cases)

PD1 ROSETTES IN VARIANT IMMUNOARCHITECTURAL PATTERNS:

Of the 34 biopsies in which immunohistochemical markers were done, 1 biopsy was not assigned a pattern as it was a core biopsy. Of the 33 cases, PD1 did not work in 2 of the cases. The staining pattern of PD1 in each of the 6 patterns of NLPHL is depicted in Table.21.

Table.21: Percentage of PD1 rosettes in the variant immunoarchitectural patterns of NLPHL

PD1 ROSETTES	A	B	C	D	E	F	TOTAL
FREQUENT (≥6%)	13(93%)	3(100%)	1(100%)	8(73%)	0	0	25
INFREQUENT (1%-5%)	0	0	0	0	0	1(100%)	1
ABSENT	1(7%)	0	0	3(27%)	1(100%)	0	5
TOTAL	14	3	1	11	1	1	31

PD1 showed frequent staining in the Classic nodular pattern (93%) followed by the T cell rich nodular pattern (73%). All cases of Serpiginous/interconnected nodular pattern and Nodular pattern with prominent extra nodular L&H cells had frequent PD1 rosettes. Infrequent PD1 rosettes were seen in only moth eaten pattern with B cell rich background. The overall observation was that the nodular patterns had a higher frequency of staining than the diffuse variants which showed only infrequent or absent staining.

CD57 ROSETTES IN VARIANT IMMUNOARCHITECTURAL PATTERNS:

The staining pattern of CD57 in the 33 biopsies assigned to the 6 patterns is depicted in Table.22.

Table.22: Percentage of CD57 rosettes in the variant immunoarchitectural patterns of NLPHL

CD57 ROSETTES	A	B	C	D	E	F	TOTAL
FREQUENT (≥6%)	12(80%)	1(33%)	1(100%)	5(42%)	0	0	19
INFREQUENT (1%-5%)	2(13%)	1(33%)	0	4(33%)	0	0	7
ABSENT	1(7%)	1(33%)	0	3(25%)	1(100%)	1(100%)	7
TOTAL	15	3	1	12	1	1	33

CD57 showed frequent rosettes the Nodular pattern with prominent extra nodular L&H cells (100%), Classic nodular pattern (80%), T cell rich nodular pattern (42%) and Serpiginous/interconnected nodular pattern (33%). Patterns D, A and B showed infrequent CD57 rosettes also. Diffuse pattern (T cell rich B cell lymphoma like) and moth eaten pattern with B cell rich background lacked CD57 rosettes in all cases. The overall observation was that the nodular patterns had a higher frequency of staining than the diffuse variants which showed only infrequent or absent staining.

Pattern E lacked PD1and CD57 rosettes and included only one case. Hence we could not evaluate the efficacy of the 2 immunomarkers in the diffuse pattern (T cell rich B cell lymphoma like) NLPHL.

PD1 AND CD57 IN THRLBCL:

In our study, 10 cases of THRLBCL were included as controls.

PD1 rosettes were seen in only 1 of 10 cases (10%) and were infrequent. The remaining 9 of 10 cases showed absent staining for PD1 in the presence of positive internal and external controls. (Fig.9)

CD57 rosettes were seen infrequently in 2 of 10 cases (20%) and the remaining cases (80%) lacked CD57 rosettes. (Fig.10)

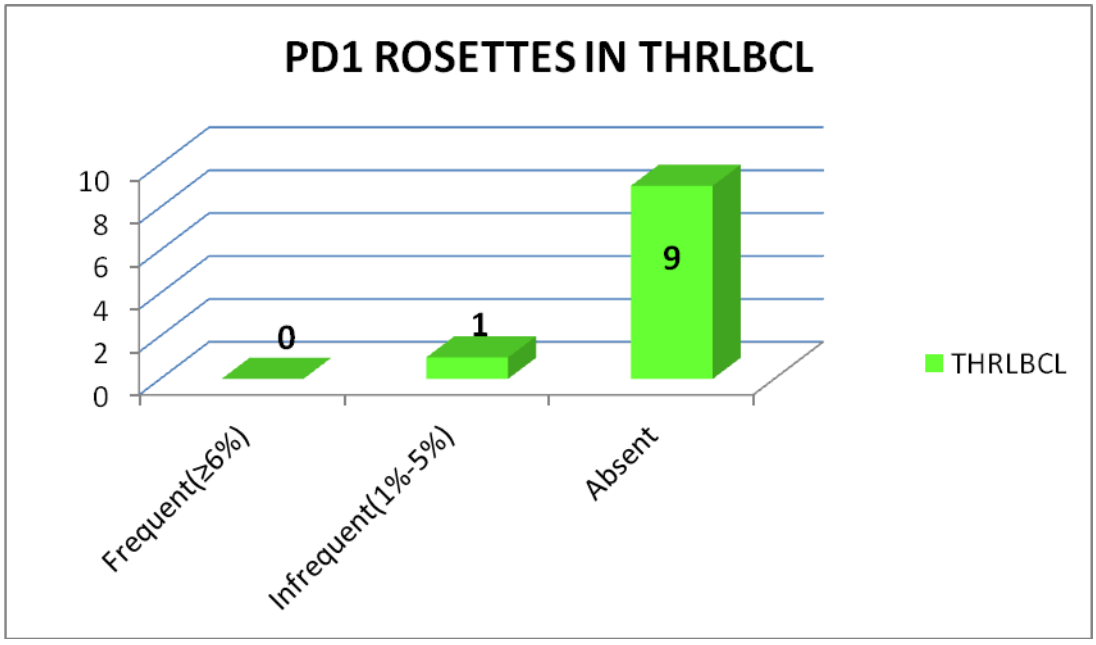


Figure.9: PD1 rosettes in THRLBCL (x axis: Percentage of PD1 rosettes, y axis: No. of cases)

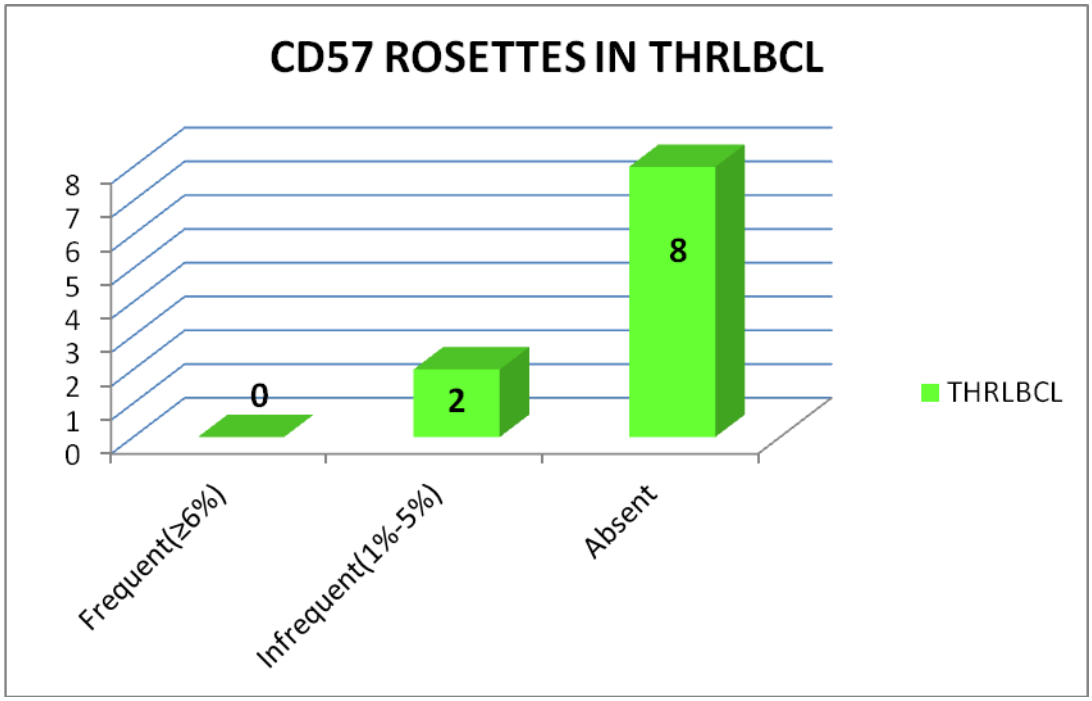


Figure.10: CD57 rosettes in THRLBCL (x axis: Percentage of CD57 rosettes, y axis: No. of cases)

SENSITIVITY AND SPECIFICITY OF PD1 AND CD57:

PD1:

The sensitivity and specificity of PD1 was assessed by comparing the staining between 32 biopsies of NLPHL and 10 biopsies of THRLBCL.

26 / 32 biopsies (81.25%) of NLPHL showed PD1 rosettes in contrast to 1 / 10 (10%) cases of THRLBCL. The sensitivity of PD1 was found to be 81.3% (confidence interval-95% CI: 63.6%-92.8%) and specificity was 90% (95% CI: 55.5%-99.7%).

The positive likelihood ratio of PD1 is 8.13(95%CI: 1.26-52.6), i.e., if PD1 rosettes are present, there is 8.13 times more likely chance that the patient has NLPHL rather

than THRLBCL. The positive predictive value of PD1 in the diagnosis of NLPHL was found to be 96.3%, i.e., 96.3% of patients who have PD1 positive rosettes actually have NLPHL rather than THRLBCL and the negative predictive value of PD1 was found to be 60%, i.e., 60% of the patients in whom PD1 rosettes are absent do not have NLPHL. (Table.23)

Table.23: Comparison of frequency of PD1 rosettes in NLPHL and THRLBCL

	PD1 ROSETTES		
DISEASE	**POSITIVE**	**NEGATIVE**	**TOTAL**
NLPHL	26 (81.25%)	6 (18.75%)	32
THRLBCL	1 (10%)	9 (90%)	10
TOTAL	27	15	42

CD57:

26 / 34 biopsies (76.47%) of NLPHL showed CD57 rosettes in contrast to 2 / 10 biopsies (23.53%) of THRLBCL. The sensitivity of CD57 was found to be 76.5% (95% CI: 44.4%-97.5%) and specificity was 80% (95% CI: 44.4%-97.5%).

The positive likelihood ratio of CD57 is 3.82 (95%CI: 1.09-13.4), i.e., if CD57 rosettes are present, there is only 3.82 times more likely chance that the patient has NLPHL rather than THRLBCL. The positive predictive value of CD57 in the diagnosis of NLPHL was found to be 92.9%, i.e., 92.9% of patients who have CD57 positive rosettes actually have NLPHL rather than THRLBCL and the negative

predictive value of CD57 was found to be 50%, i.e., 50% of the patients in whom CD57 rosettes are absent do not have NLPHL. (Table.24)

Table.24: Comparison of CD57 rosettes in NLPHL and THRLBCL

	CD57 ROSETTES		
DISEASE	**POSITIVE**	**NEGATIVE**	**TOTAL**
NLPHL	26 (76.47%)	8 (23.53%)	34
THRLBCL	2 (20%)	8 (80%)	10
TOTAL	28	16	44

Overall, taking into consideration the above findings, **PD1 was found to be a more sensitive and specific marker than CD57 in the diagnosis of NLPHL**.

ILLUSTRATIONS

ILLUSTRATIONS

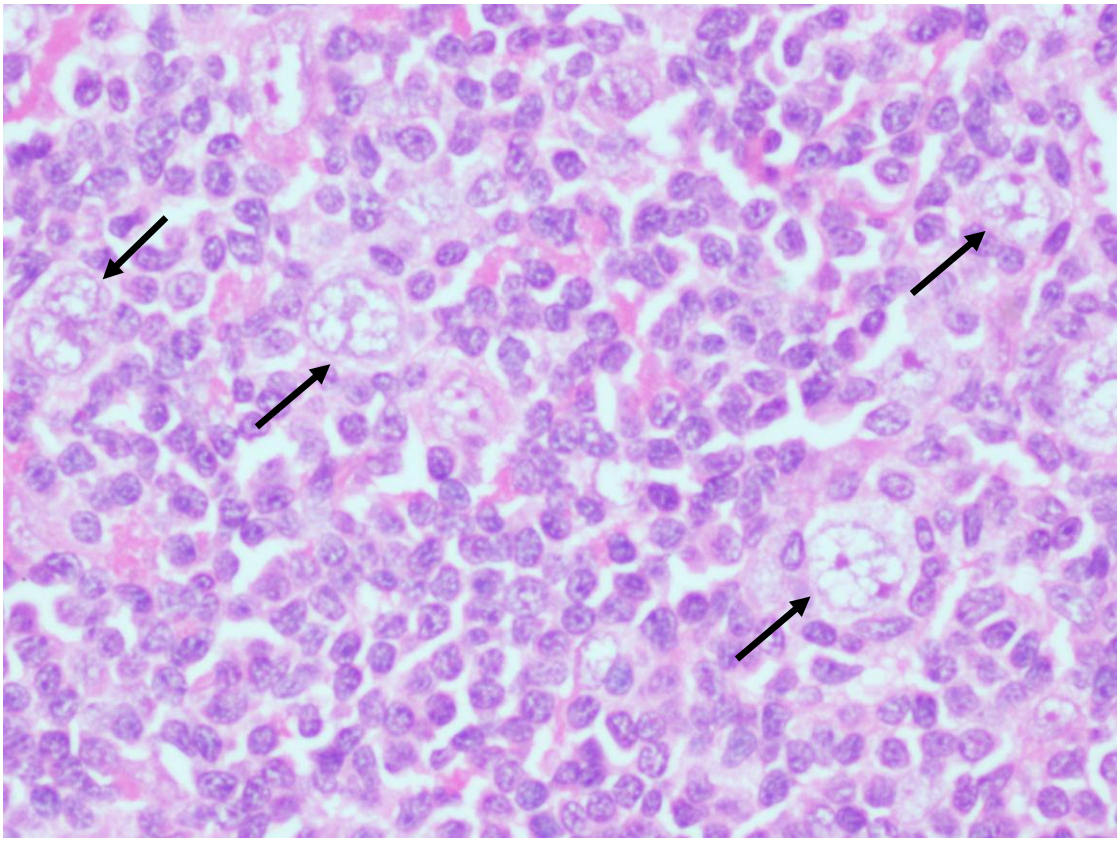


Figure 1: LP CELLS (ARROWS), H&E STAIN, 40x

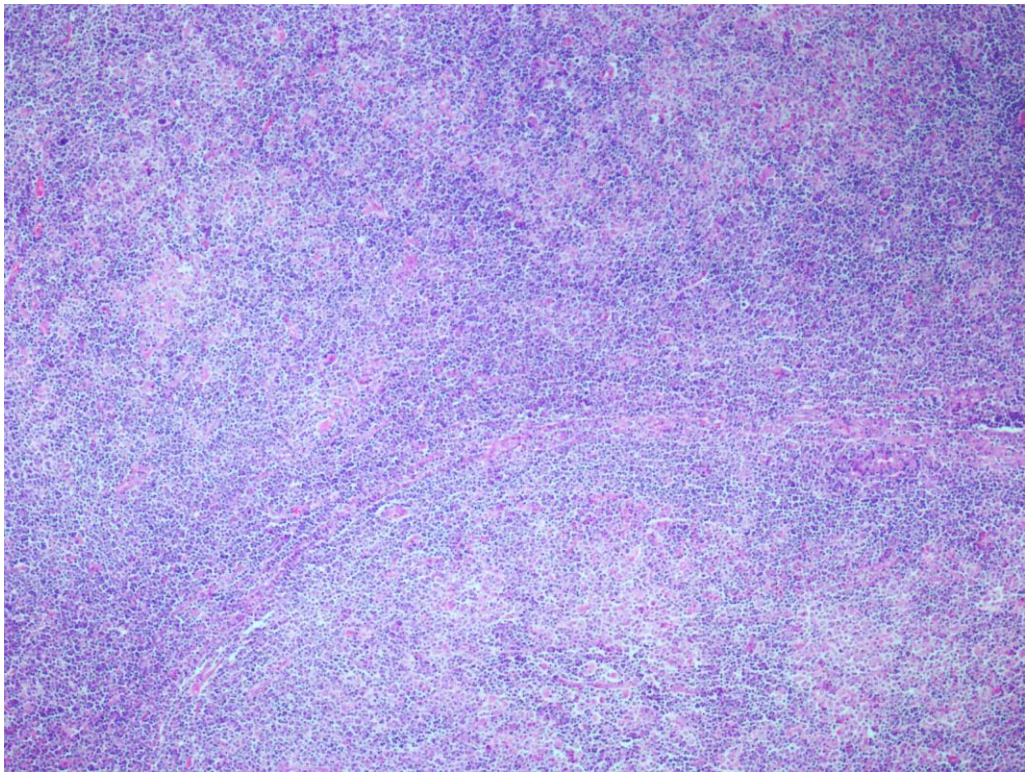


Figure 2.1: NLPHL PATTERN A (CLASSIC NODULAR), H&E STAIN, 4X

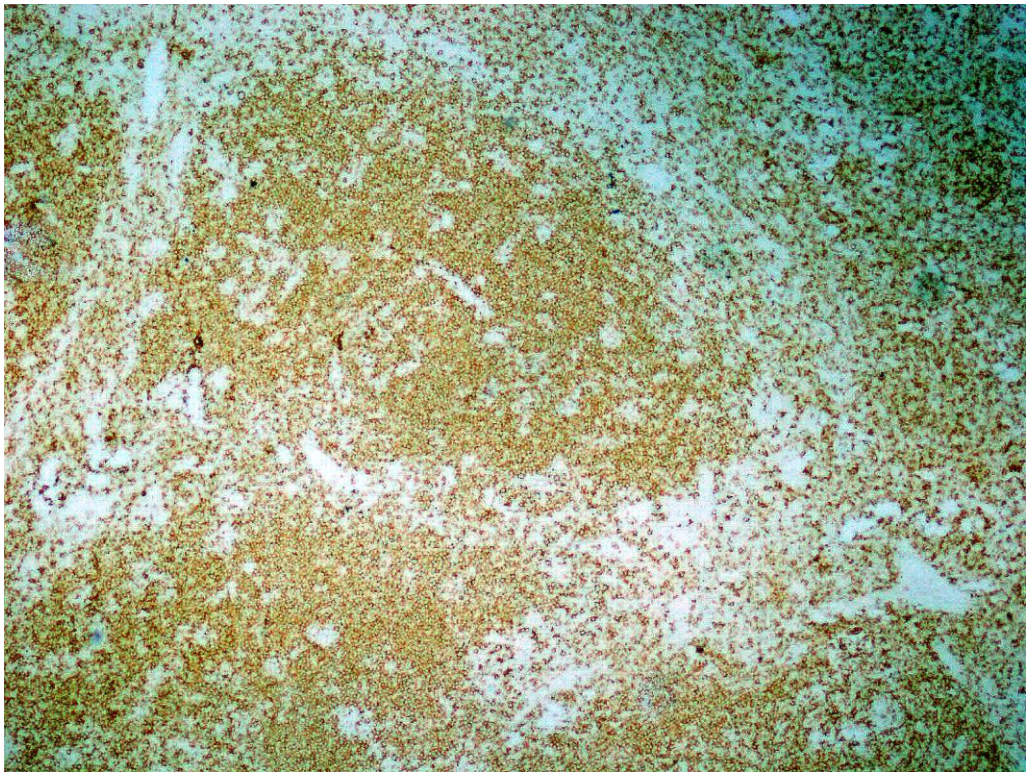


Figure 2.2: NLPHL PATTERN A, CD20 IMMUNOSTAIN, 4x

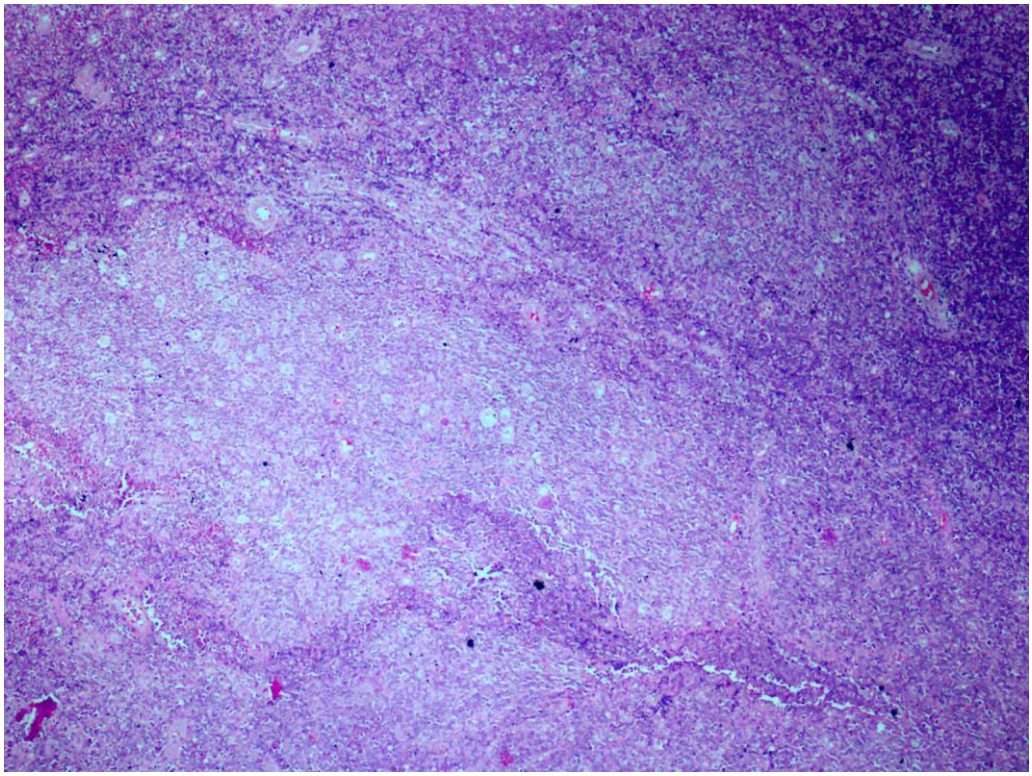


Figure 3.1: NLPHL PATTERN B (SERPIGINOUS/INTERCONNECTED NODULAR), H&E STAIN, 4x

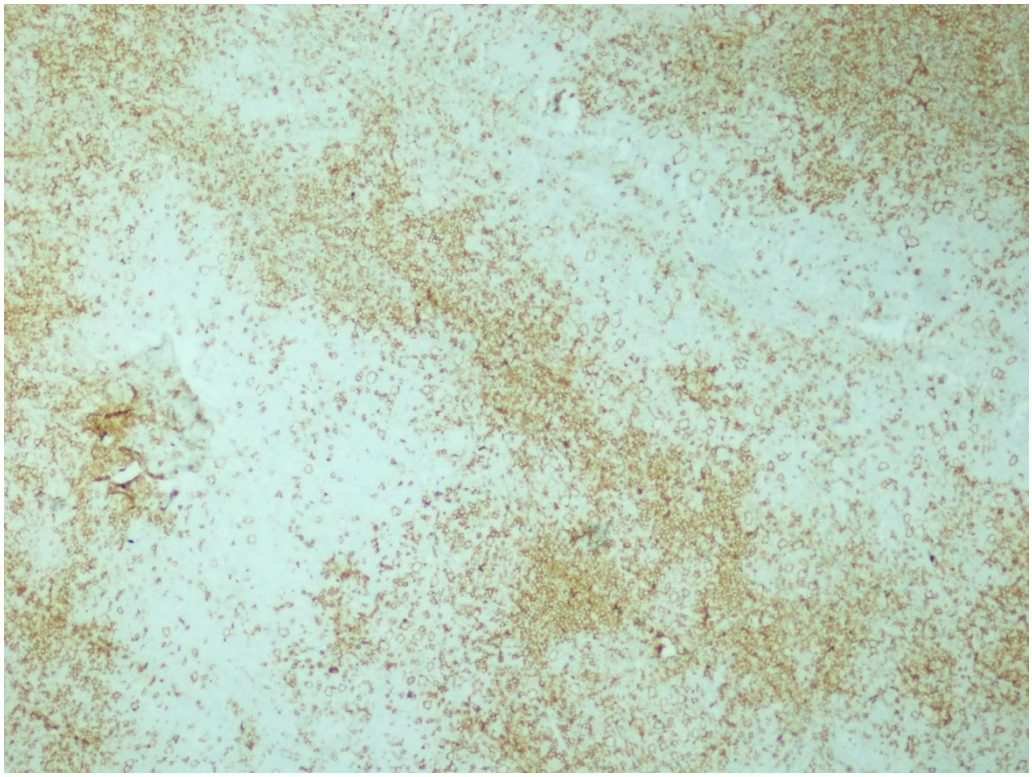


Figure 3.2: NLPHL PATTERN B, CD20 IMMUNOSTAIN, 4x

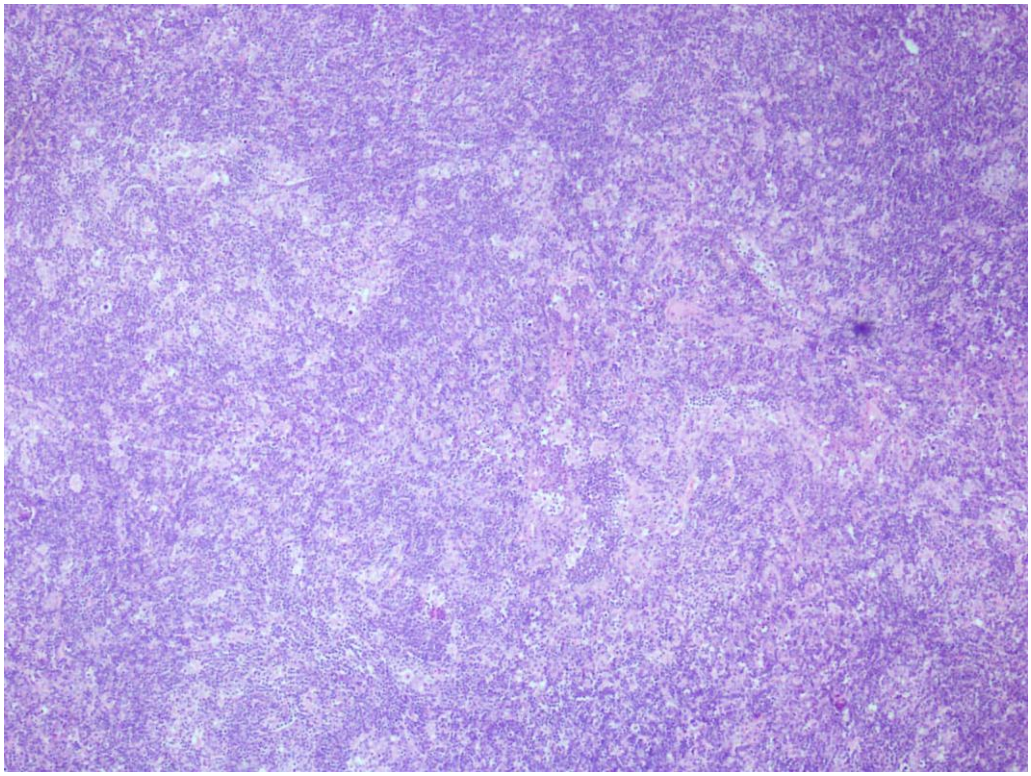


Figure 4.1: NLPHL PATTERN C, H&E STAIN, 4x

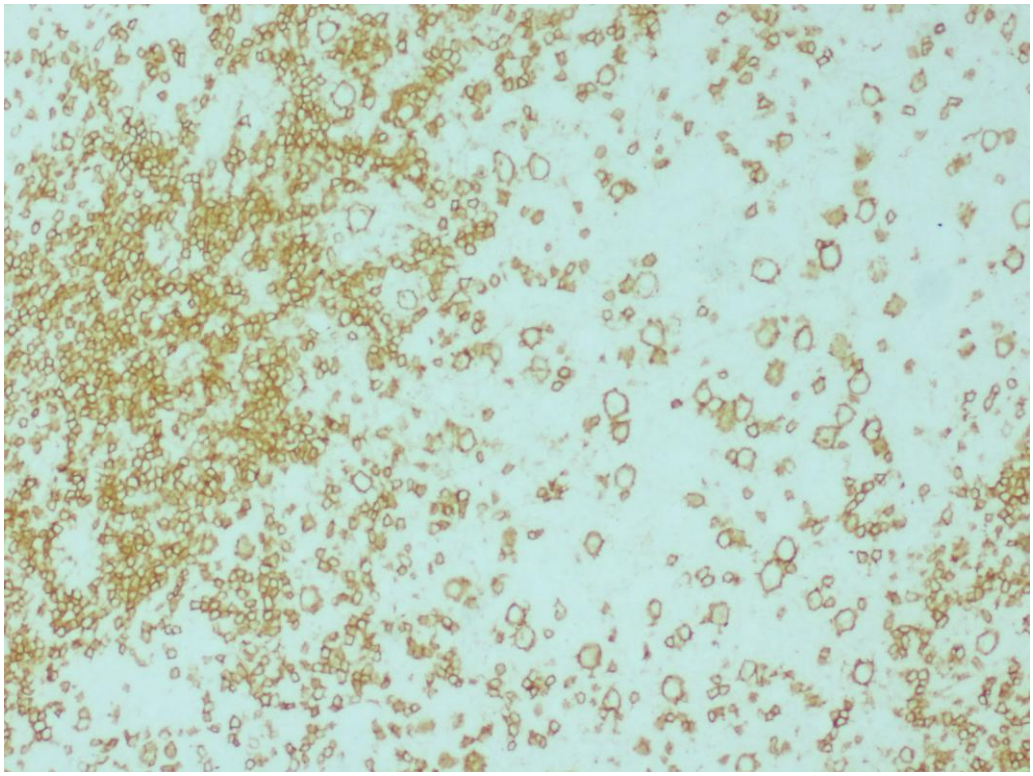


Figure 4.2: NLPHL PATTERN C (NODULAR WITH PROMINENT EXTRANODULAR LP CELLS), CD20 IMMUNOSTAIN, 10x

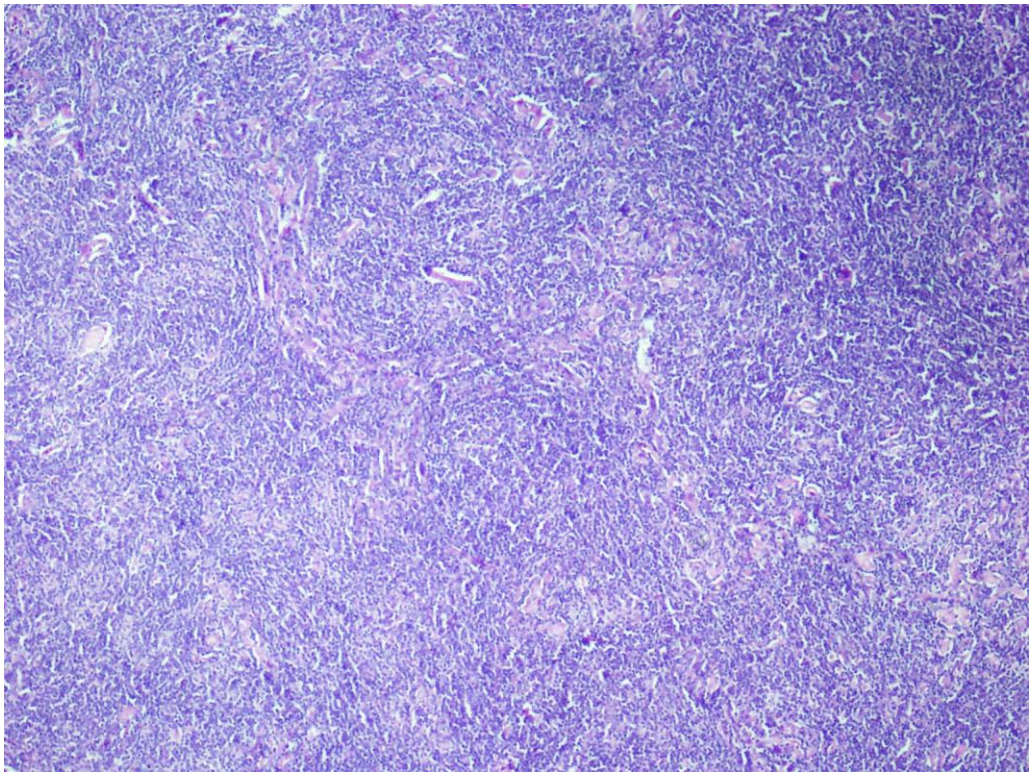


Figure 5.1: NLPHL PATTERN D (NODULAR WITH T CELL RICH BACKGROUND), H&E STAIN, 4X

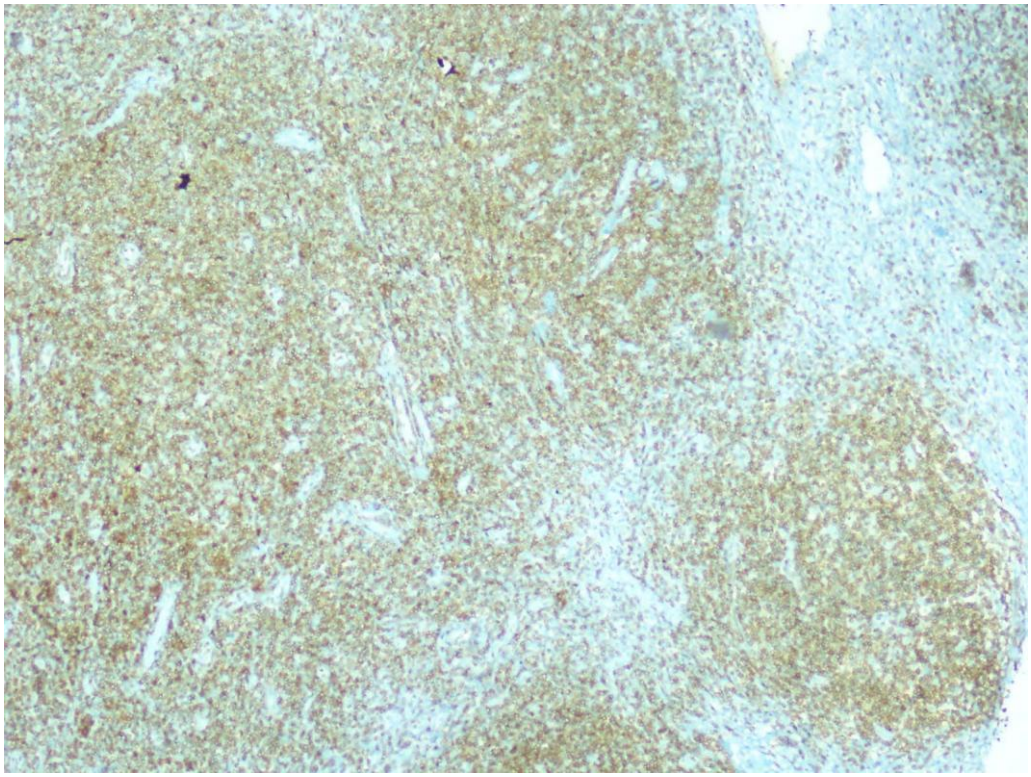


Figure 5.2: NLPHL PATTERN D, CD3 IMMUNOSTAIN, 4x

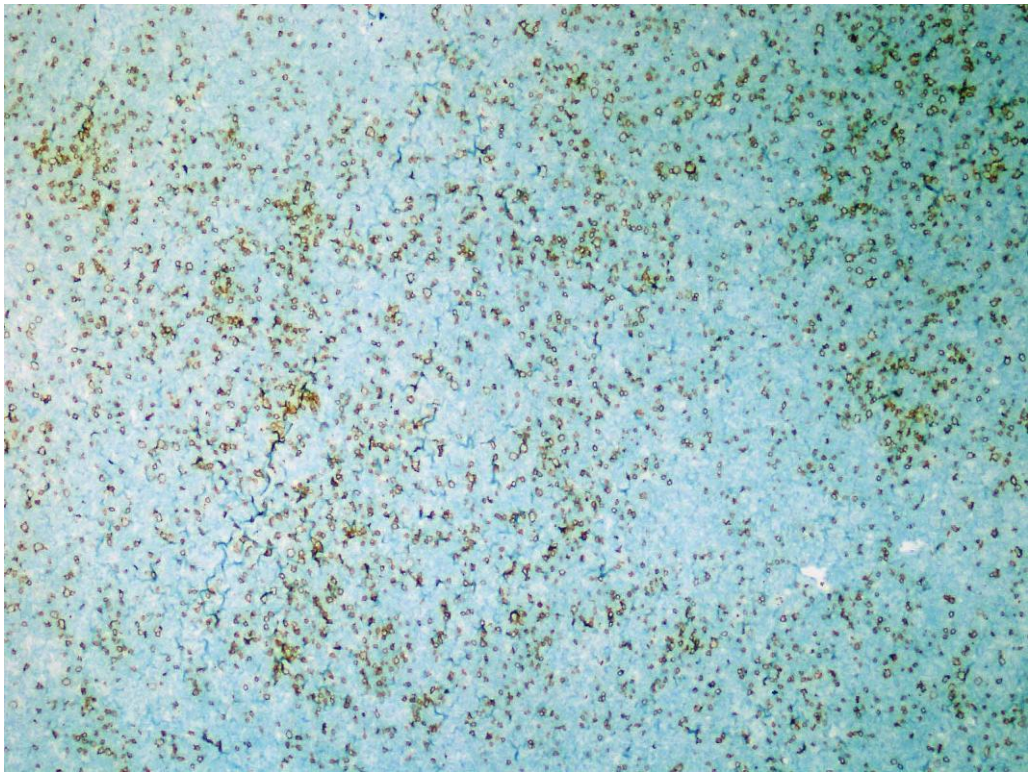


Figure 5.3: NLPHL PATTERN D, CD20 IMMUNOSTAIN, 4x

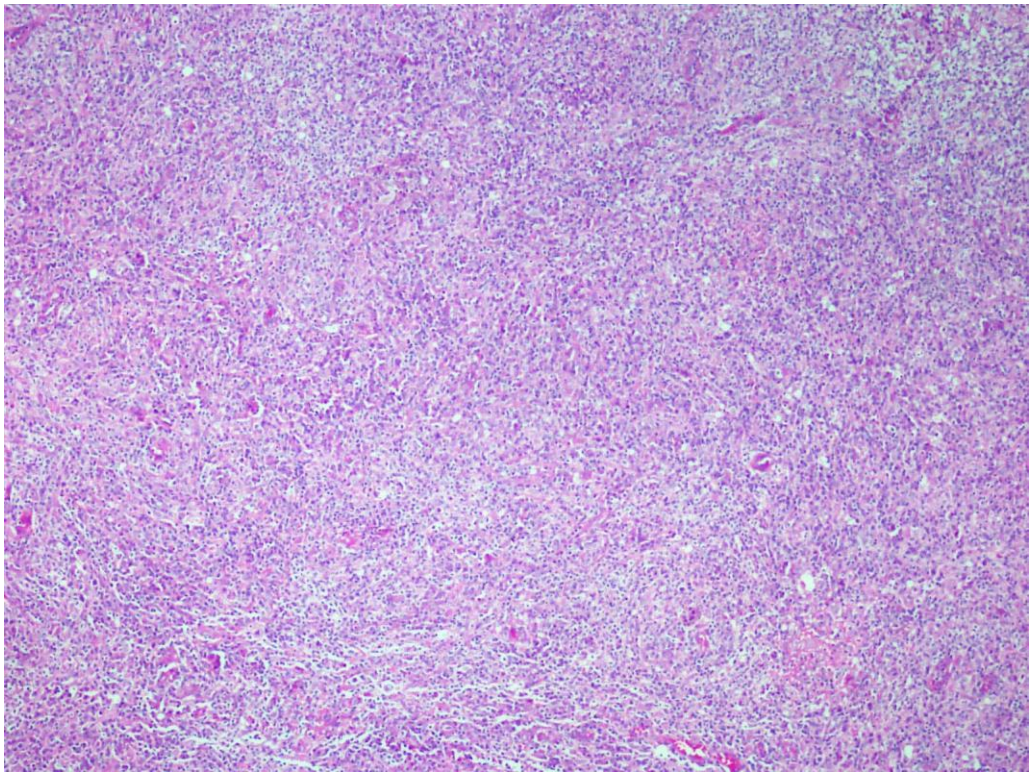


Figure 6.1: NLPHL PATTERN E (DIFFUSE TRLBCL LIKE), H&E STAIN, 4X

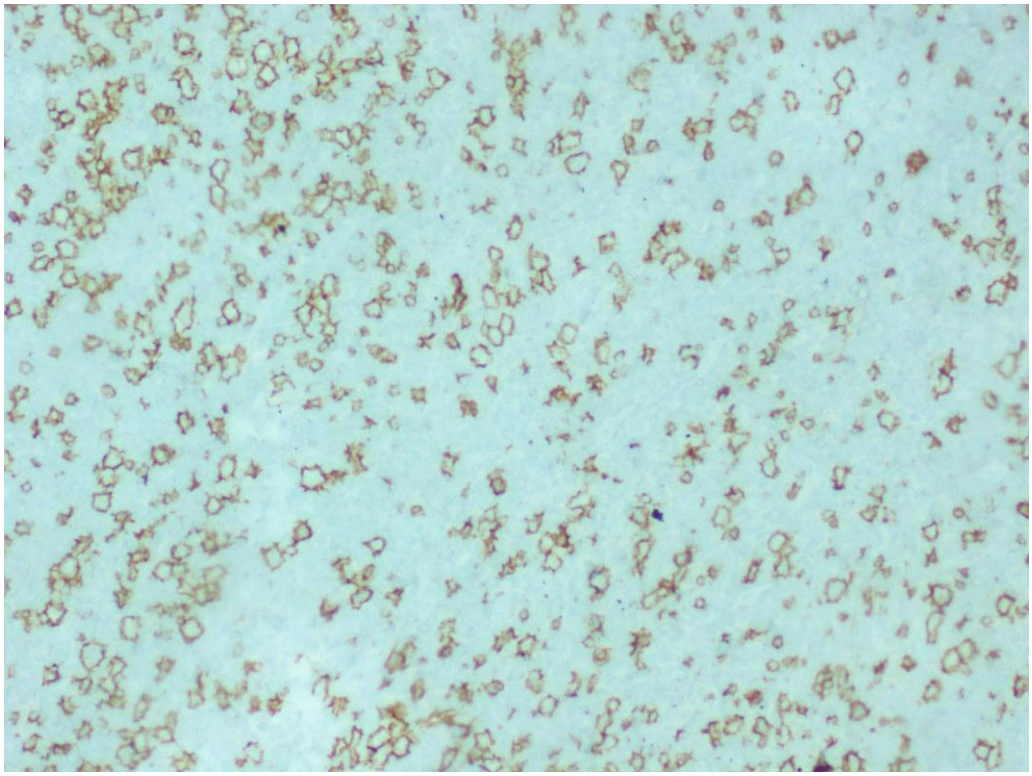


Figure 6.2: NLPHL PATTERN E, CD20 IMMUNOSTAIN, 10X

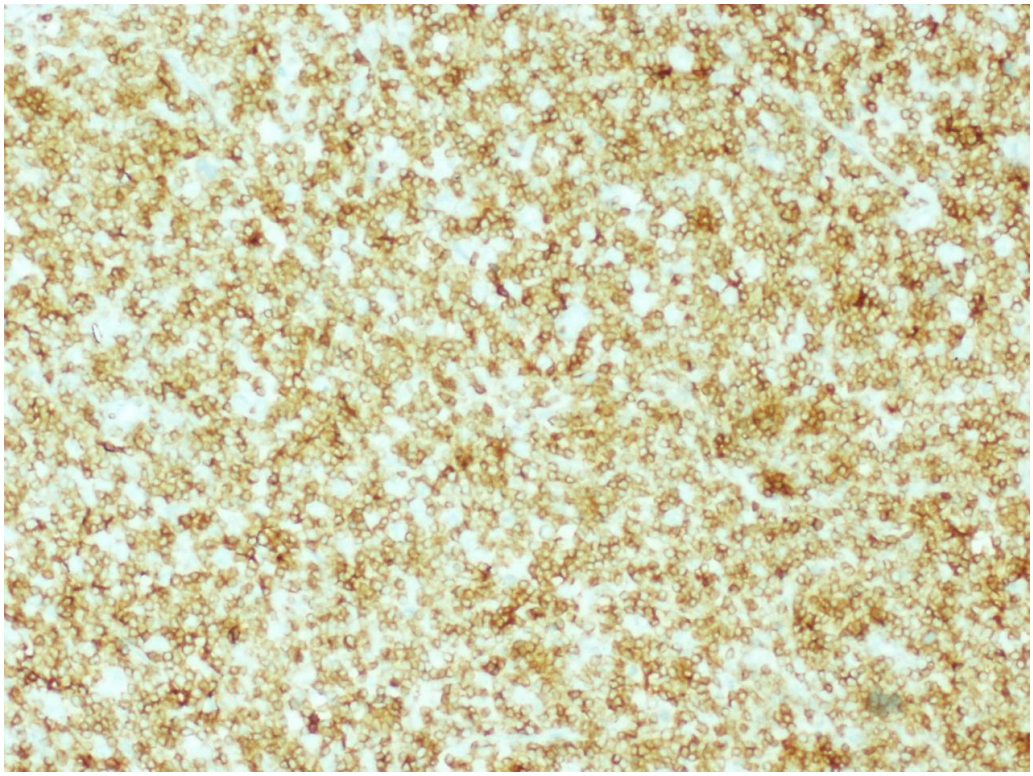


Figure 6.3: NLPHL PATTERN E, CD3 IMMUNOSTAIN, 10X

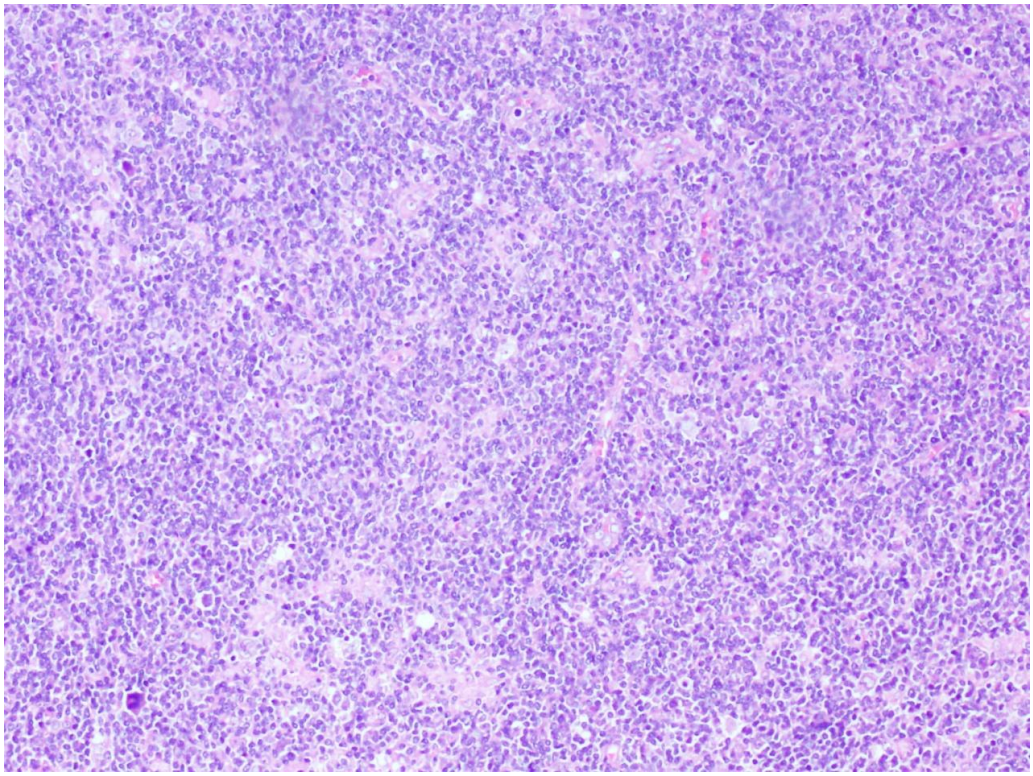


Figure 7.1:1 NLPHL PATTERN F (MOTH EATEN), H&E STAIN, 4x

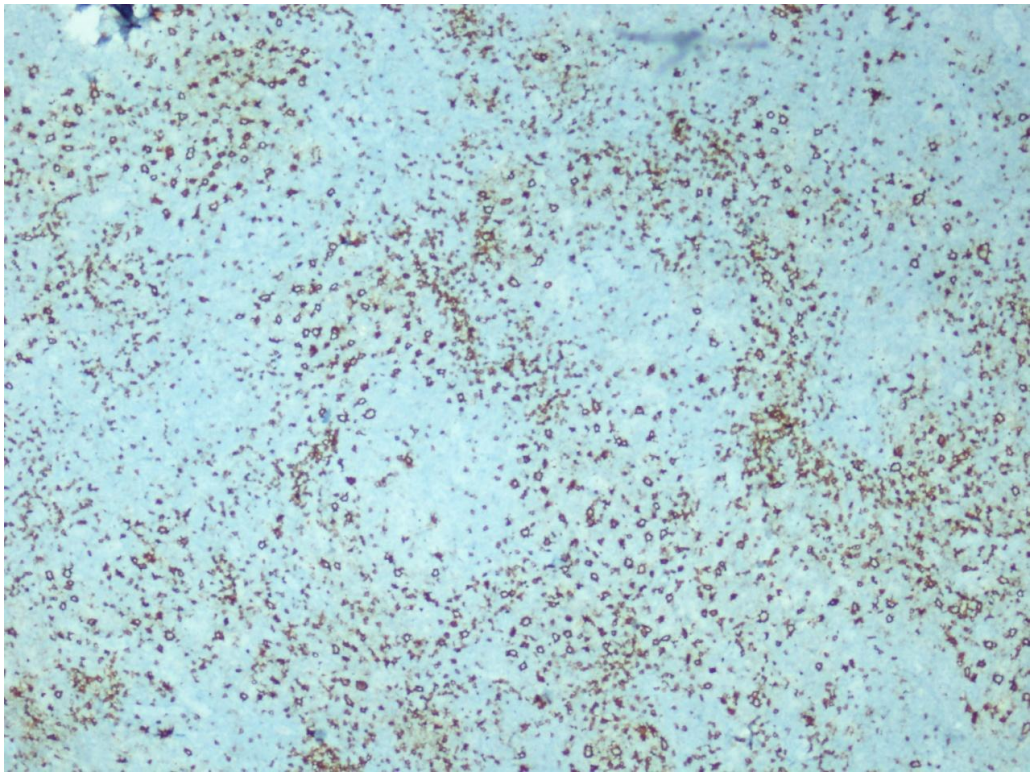


Figure 7.2: NLPHL PATTERN F, CD20 IMMUNOSTAIN, 4x

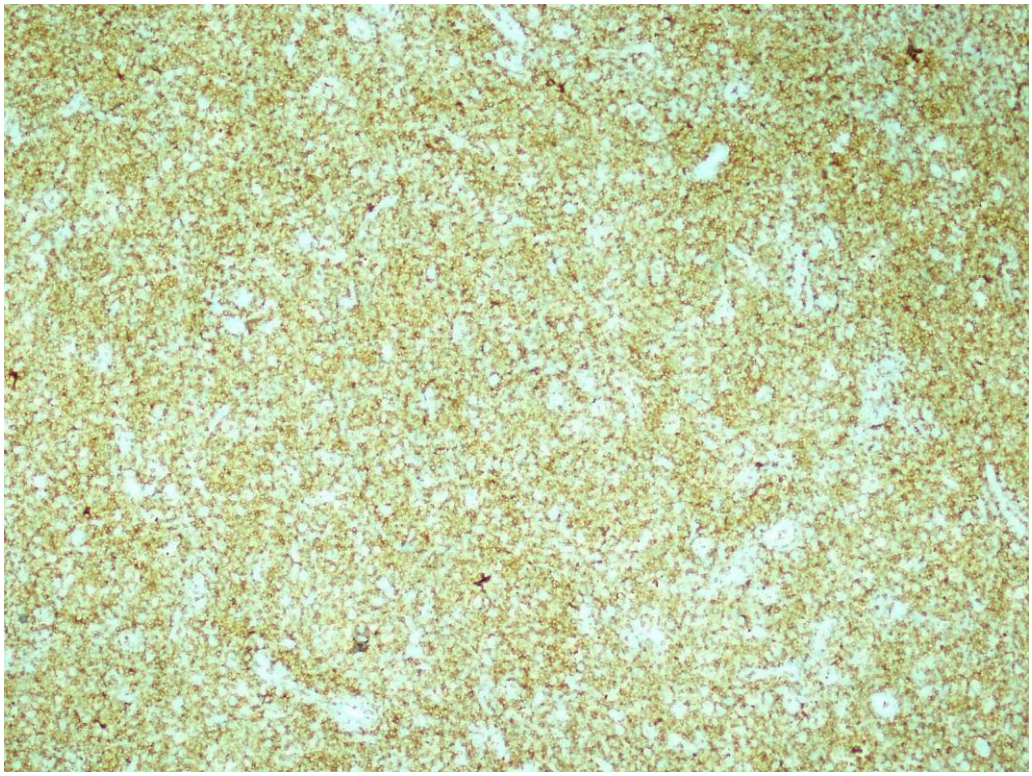


Figure 7.3: NLPHL PATTERN F, CD3 IMMUNOSTAIN, 4x

FIGURE 8: A CASE OF NLPHL WITH TRANSFORMATION TO DLBCL

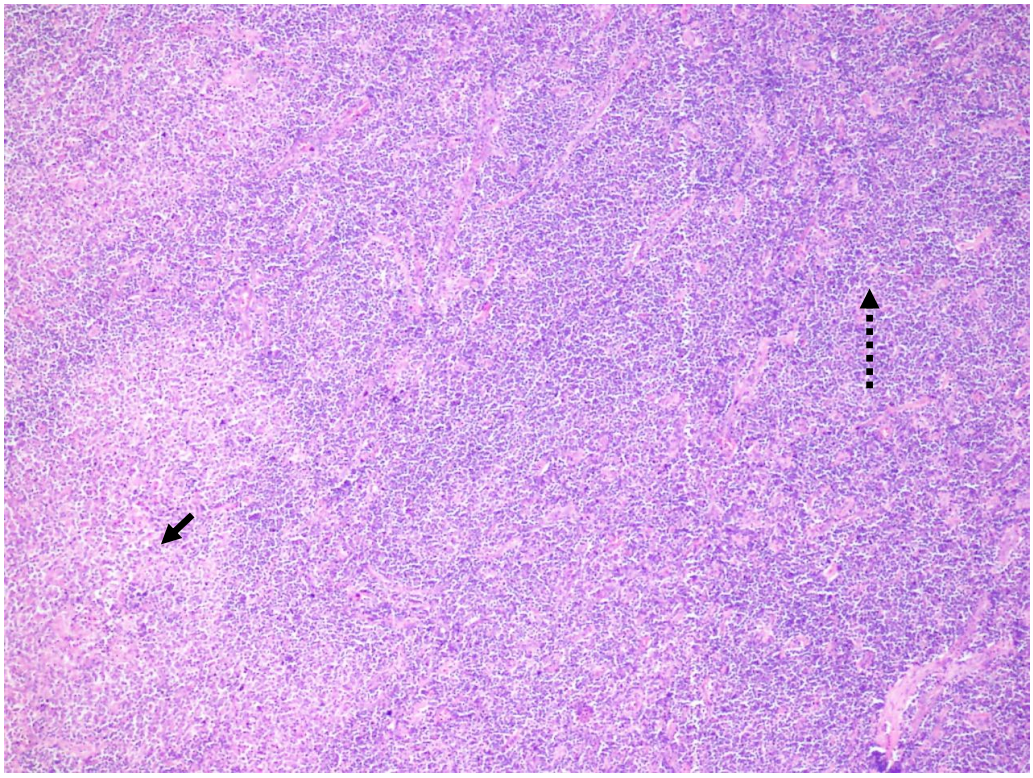


Figure 8.1, DLBCL (BOLD ARROW), NLPHL (DOTTED ARROW), H&E STAIN, 10x

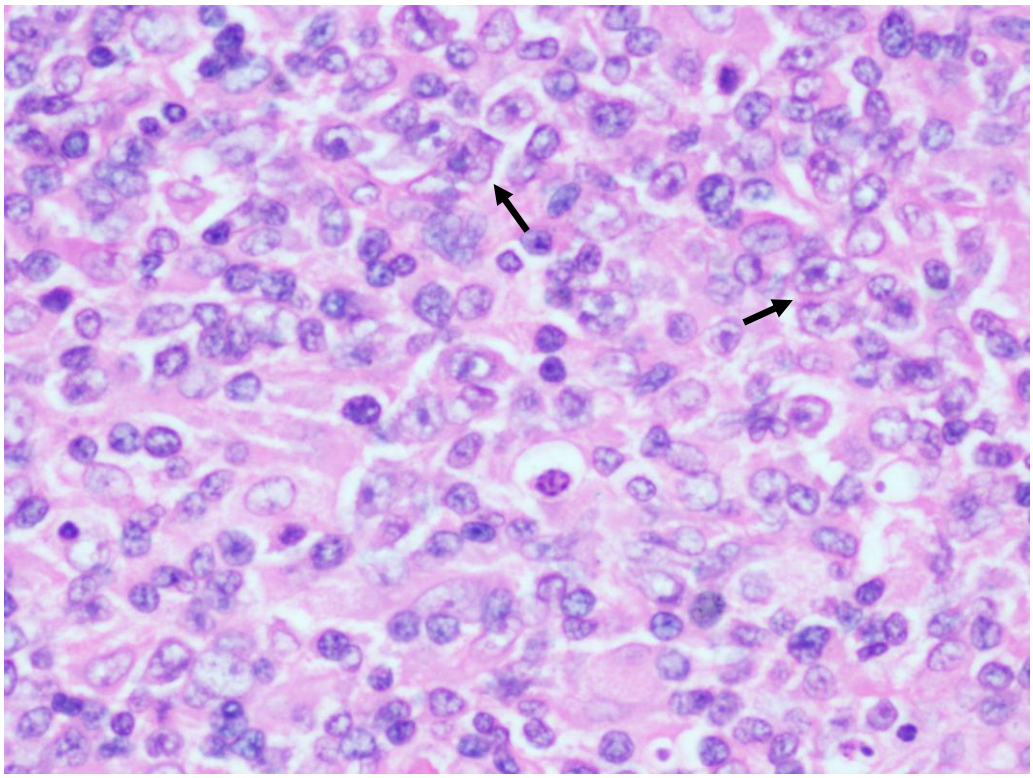


Figure 8.2: LARGE NEOPLASTIC CELLS (ARROWS), H&E STAIN, 40X

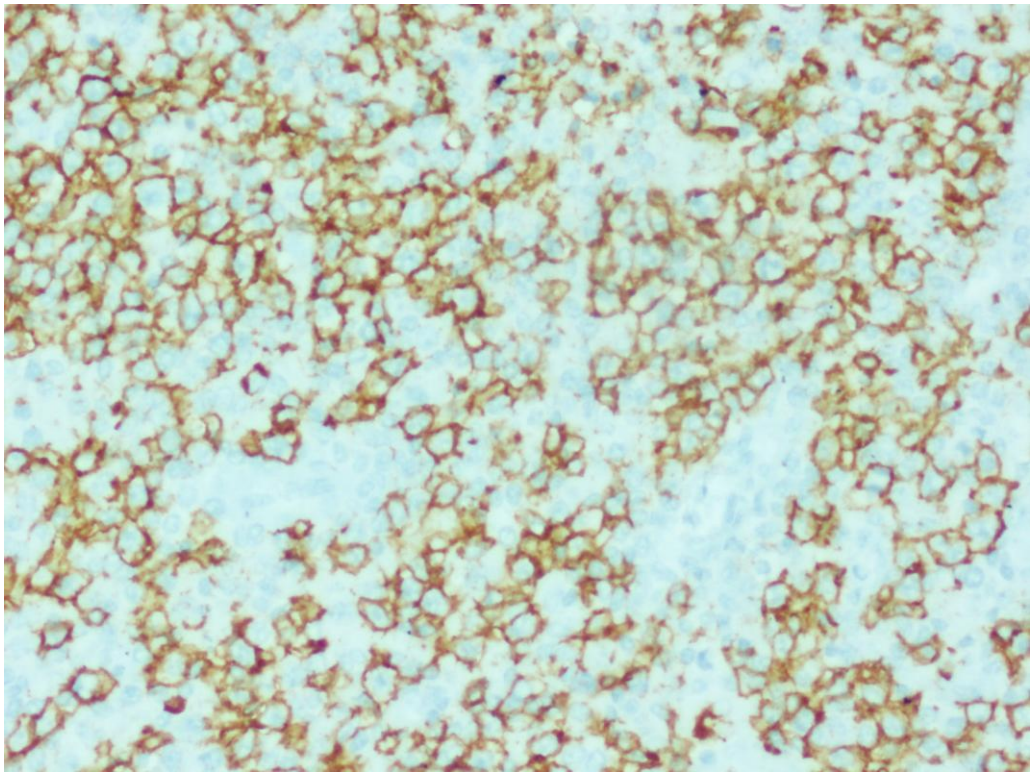


Figure 8.3: CD20 POSITIVE LARGE B CELLS, CD20 IMMUNOSTAIN, 20x

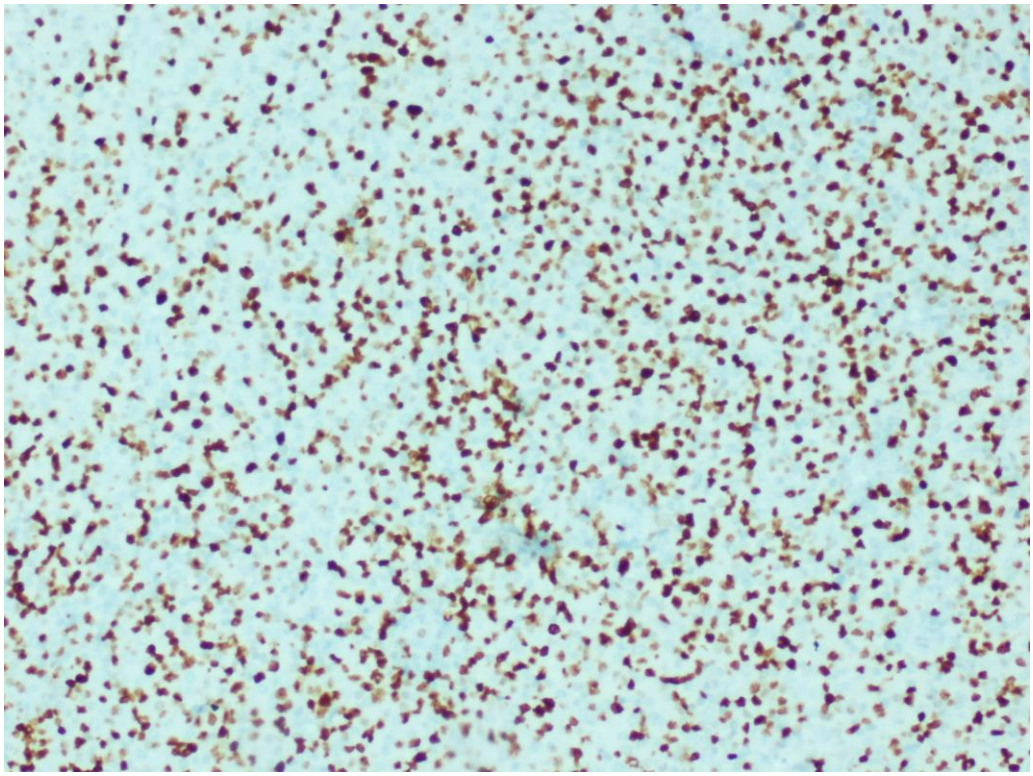


Figure 8.4: HIGH MIB-1 LABELLING INDEX IN THE DLBCL TRANSFORMATION, MIB-1 IMMUNOSTAIN, 10x

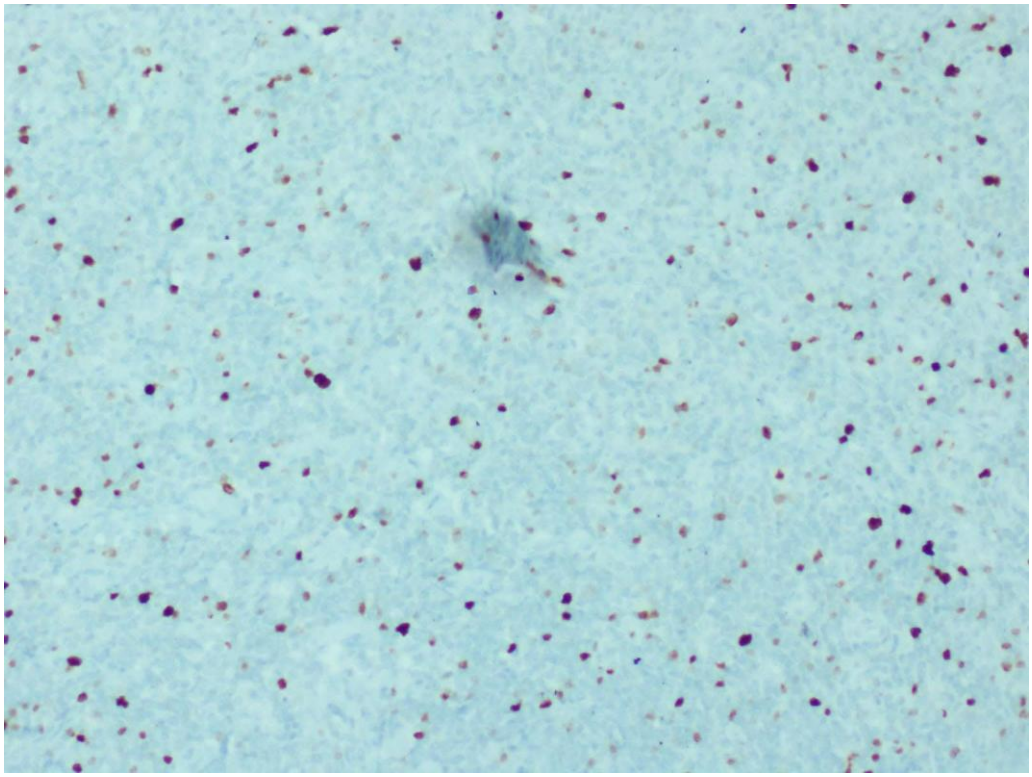


Figure 8.5: LOW MIB-1 LABELLING INDEX IN THE NLPHL AREA OF DLBCL TRANSFORMATION, MIB-1 IMMUNOSTAIN, 10x

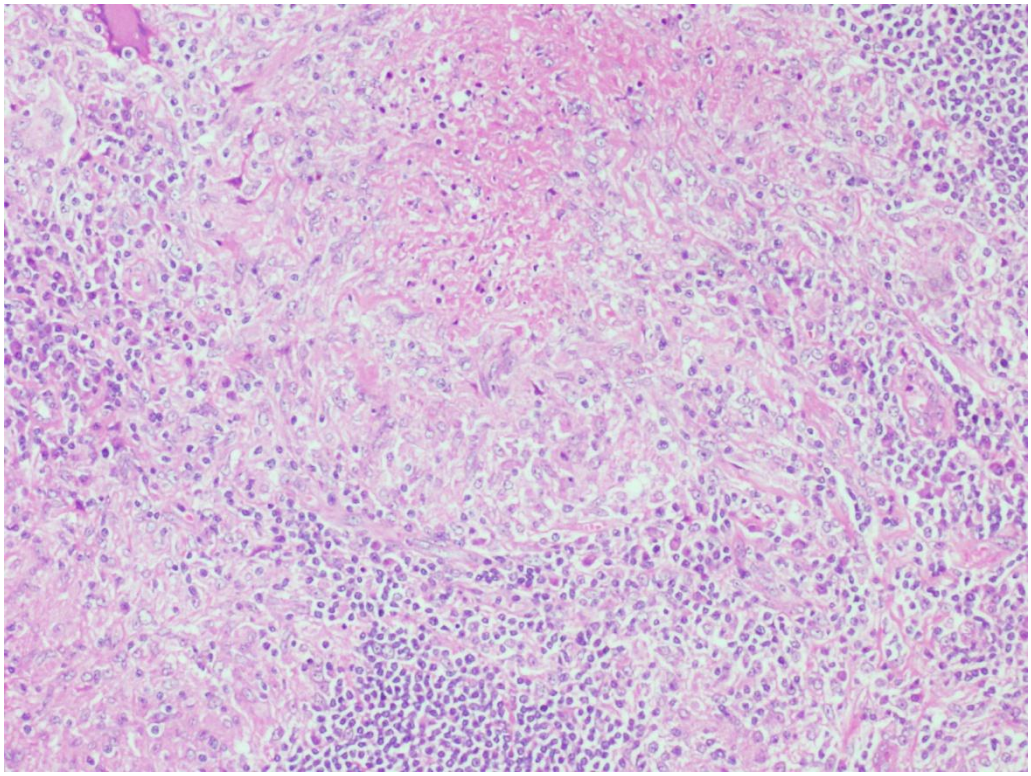


Figure 9.1: NLPHL WITH NECROTISING GRANULOMAS, H&E STAIN, 10x

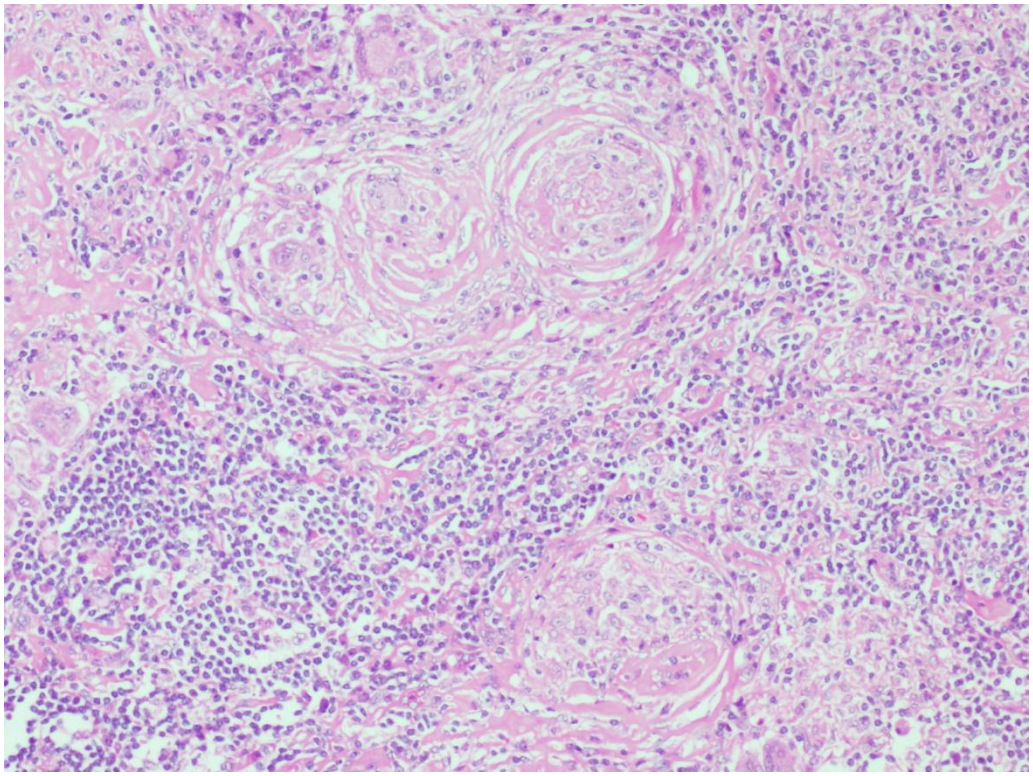


Figure 9.2: NLPHL WITH GRANULOMAS, H&E STAIN, 20x

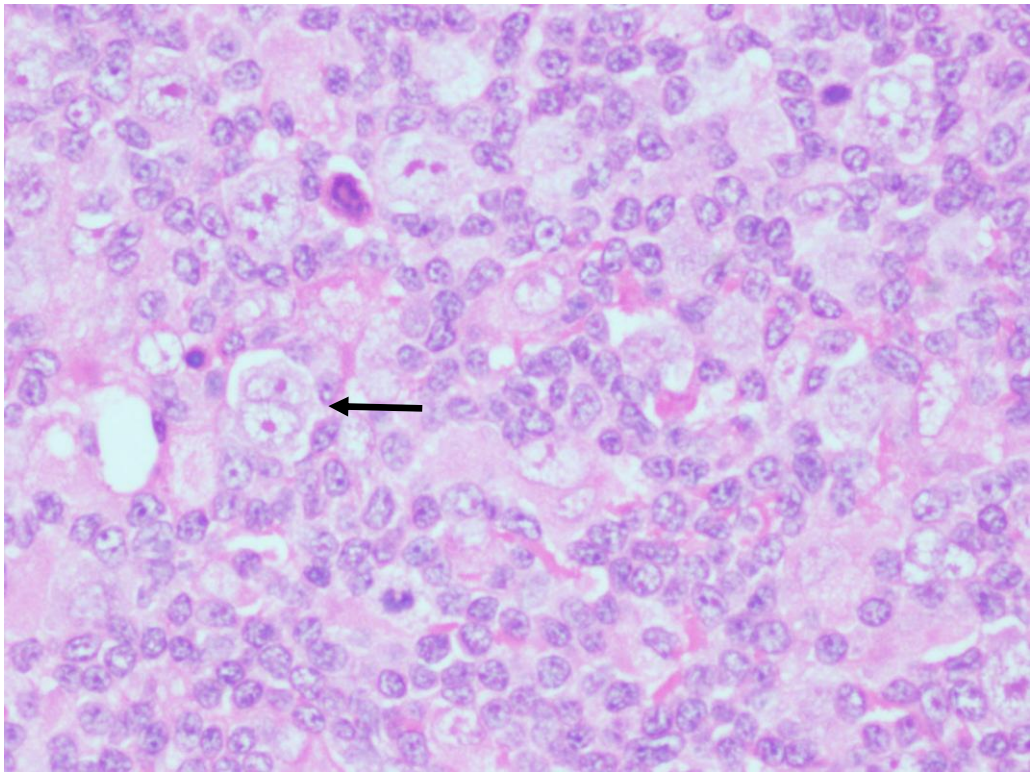


Figure 10: BINUCLEATE RS LIKE CELL (ARROW) IN NLPHL, H&E STAIN, 40x

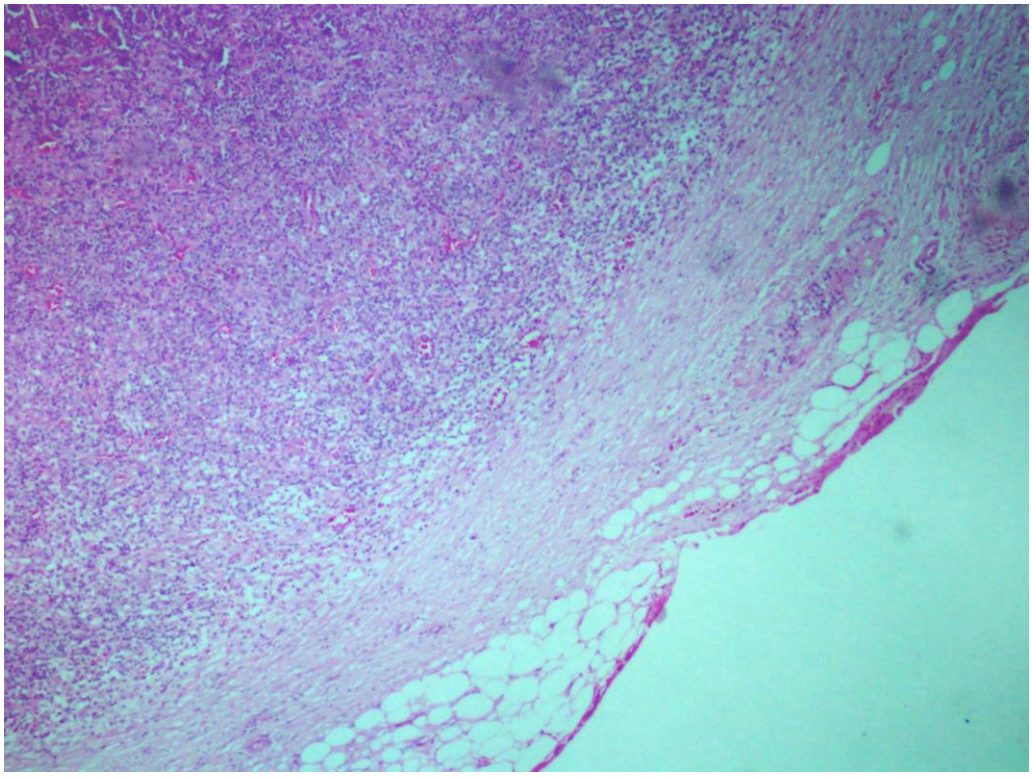


Figure 11: CAPSULAR THICKENING IN NLPHL, H&E STAIN, 4x

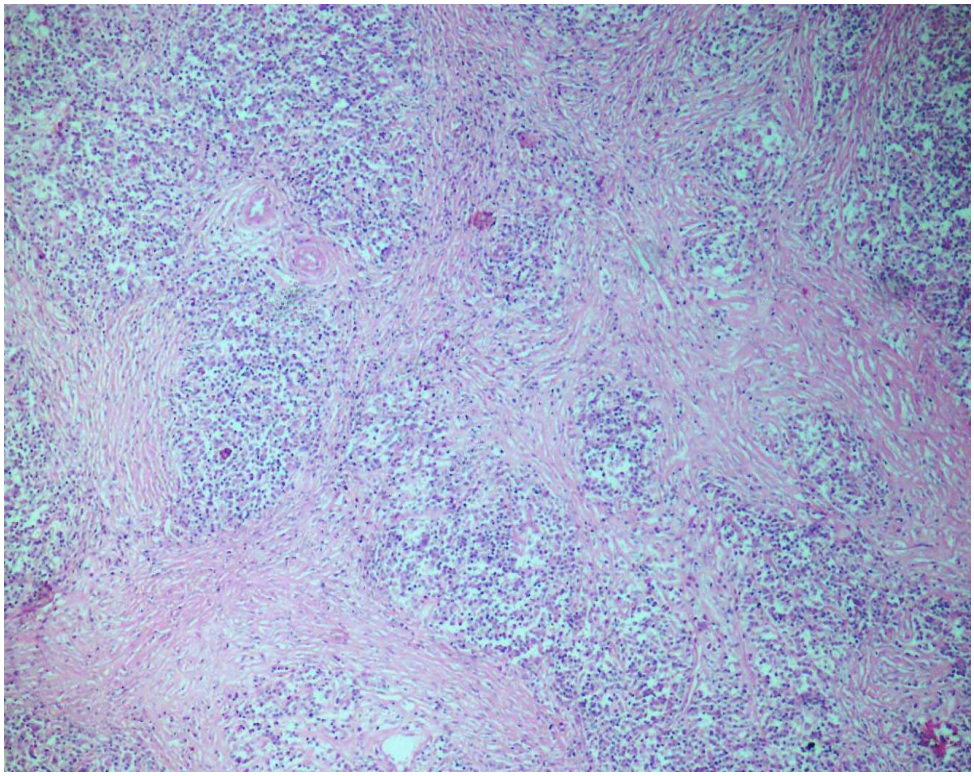


Figure 12: DENSE SCLEROTIC BANDS IN NLPHL, H&ESTAIN, 4x

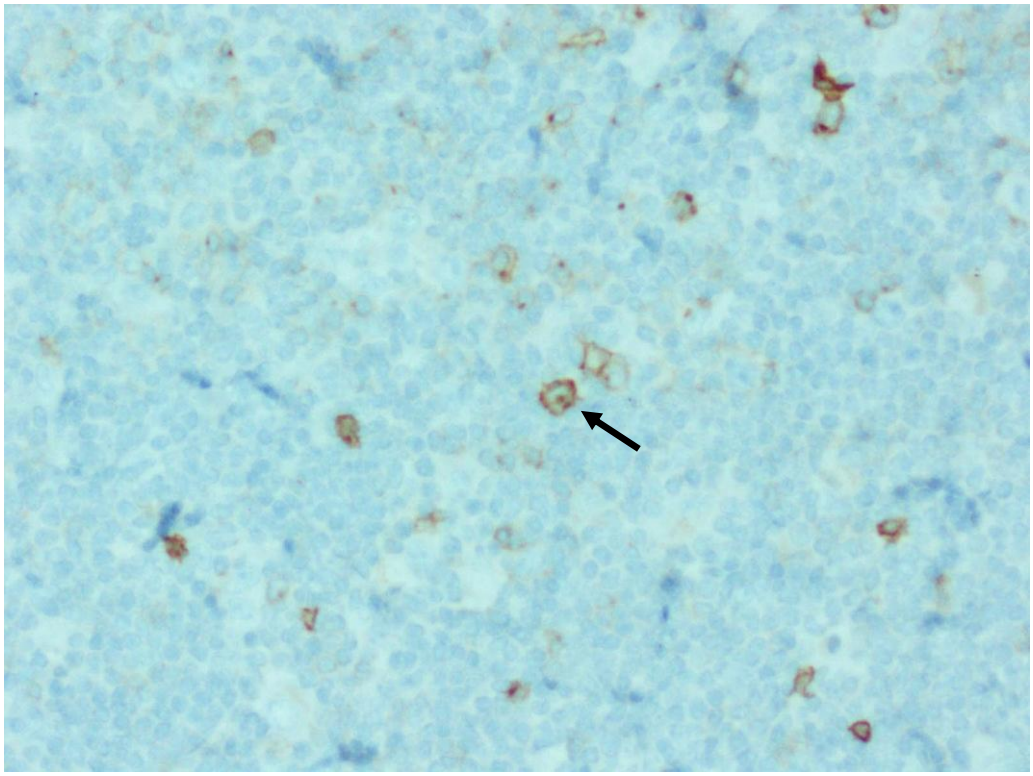


Figure 13: OCCASIONAL CD30 POSITIVE LP CELLS (ARROW) WITH MEMBRANE AND GOLGI STAINING IN NLPHL, CD30 IMMUNOSTAIN, 20x

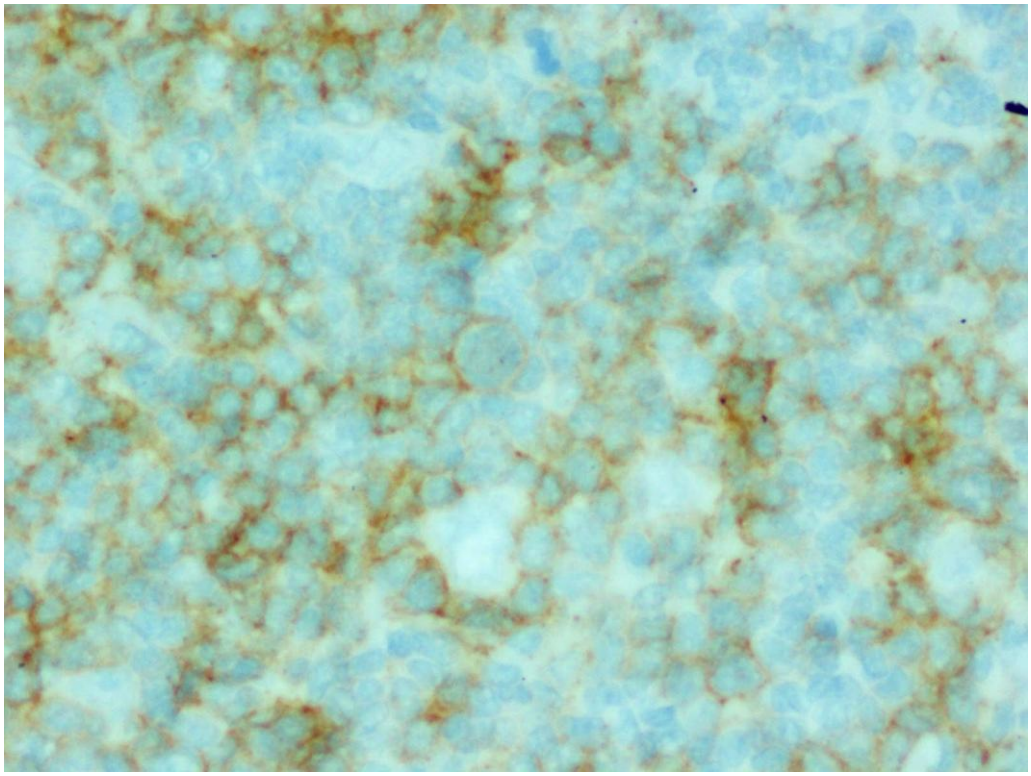


Figure 14.1: PD1 POSITIVE ROSETTE, PD1 IMMUNOSTAIN, 40x

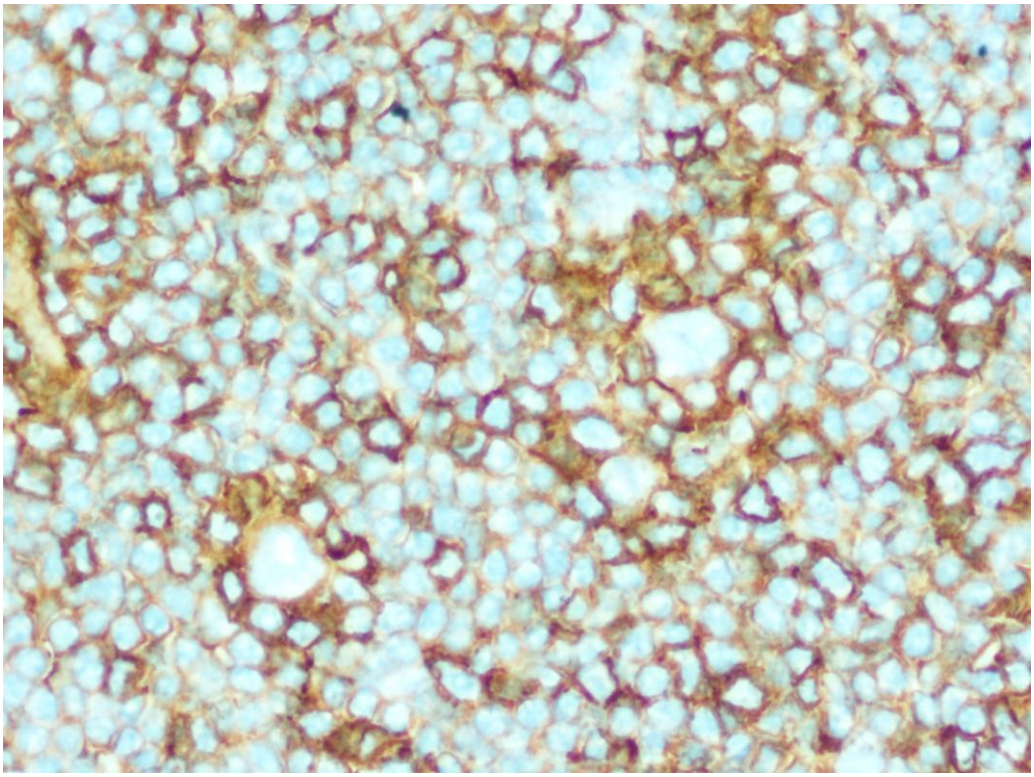


Figure 14.2: CD57 POSITIVE ROSETTES, CD57 IMMUNOSTAIN, 40x

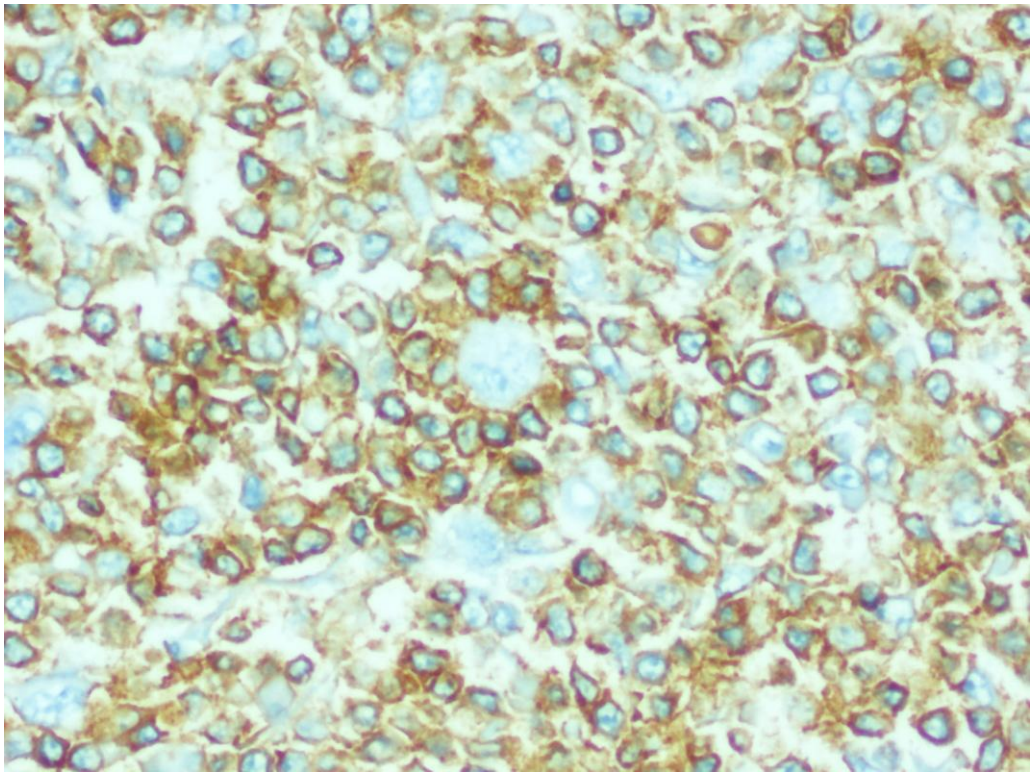


Figure 14.3: CD3 ROSETTE, CD3 IMMUNOSTAIN, 40X

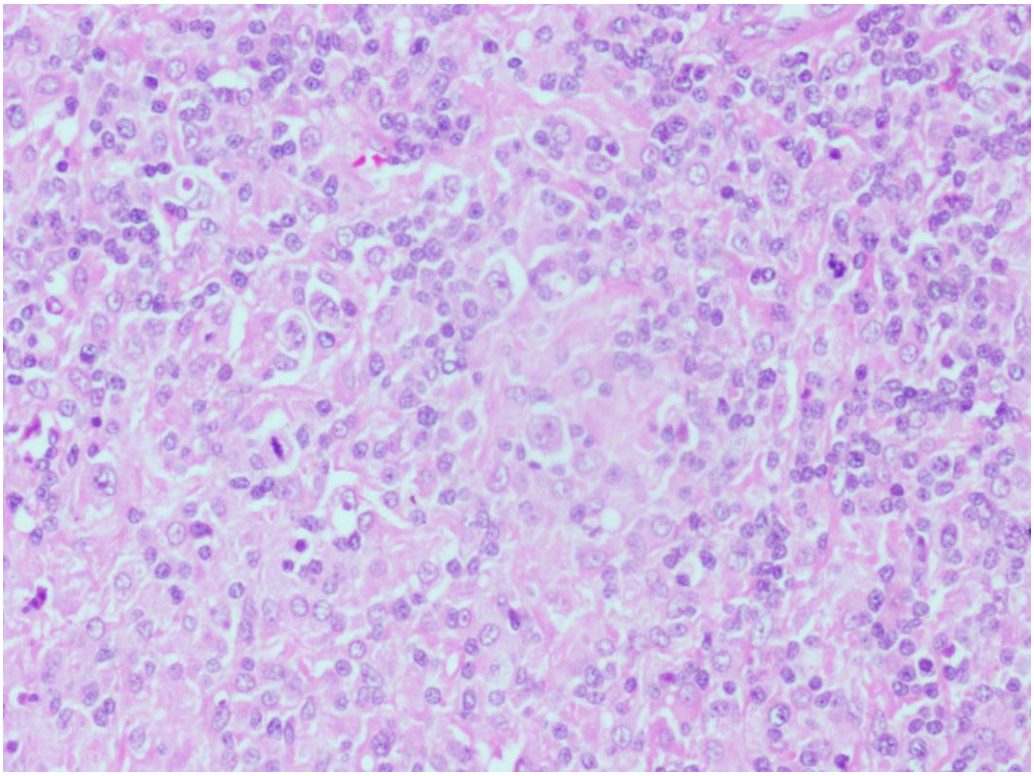


Figure 15.1: THRLBCL, H&E STAIN, 20x

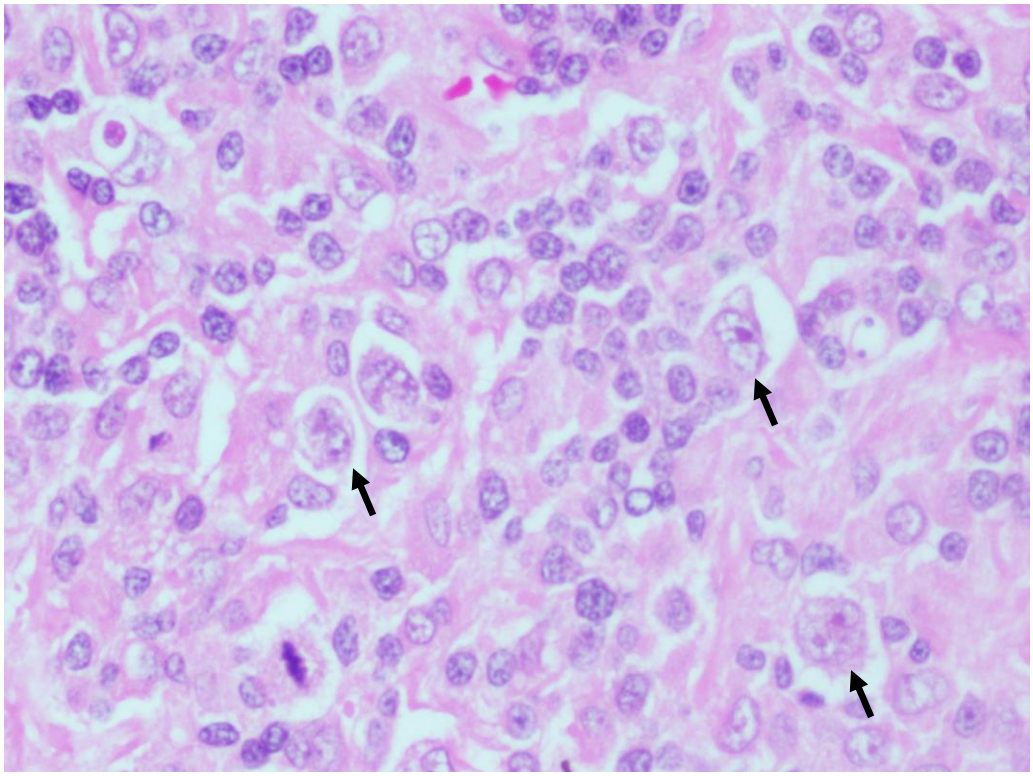


Figure 15.2: LARGE NEOPLASTIC CELLS IN THRLBCL (ARROWS), H&E STAIN, 40x

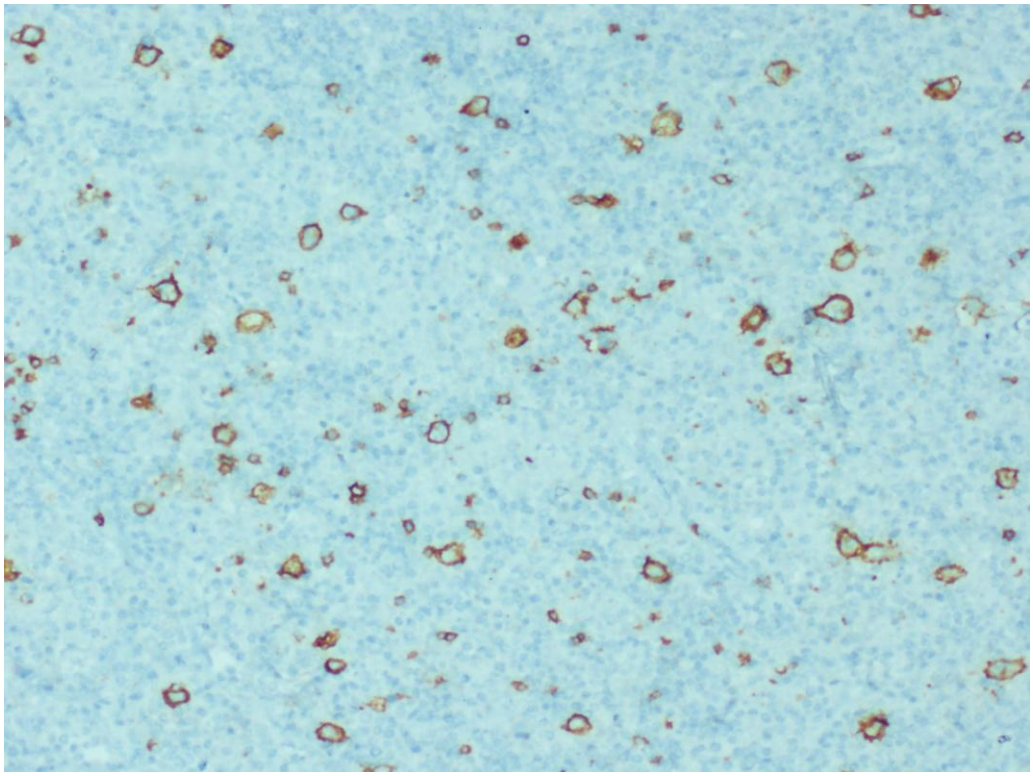


Figure 15.3: SCATTERED CD20 POSITIVE LARGE B CELLS IN THRLBCL, CD20 IMMUNOSTAIN, 10x

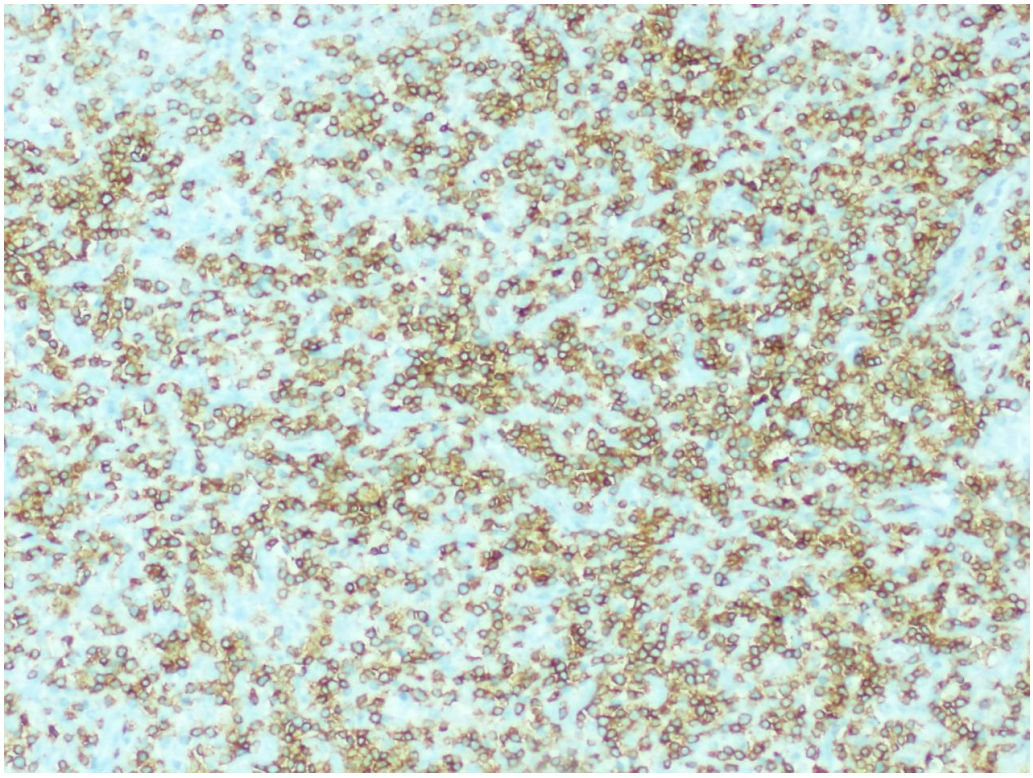


Figure 15.4: T CELL RICH BACKGROUND IN THRLBCL, CD3 IMMUNOSTAIN, 10x

DISCUSSION

DISCUSSION

Hodgkin lymphoma is broadly classified into two types: NLPHL and CHL. NLPHL is the rarer subtype and accounts for only 5%-10% of all the cases of Hodgkin lymphoma. (10) In a retrospective study conducted in an Indian cohort over a ten year period by Arora et al., NLPHL constituted 4% of all Hodgkin lymphomas. (20) The incidence rates were concordant with the incidence rates in China as well as the West. (18)(3)(19)

Traditionally, NLPHL was morphologically classified as nodular or nodular and diffuse patterns. (54) But in 2003, Fan et al., after analysing 137 biopsy samples from 118 patients diagnosed as NLPHL, described 6 variant immunoarchitectural patterns of NLPHL. The importance of sub typing NLPHL into variant immunoarchitectural patterns is that they are clinically significant. There have been only four studies till date that have taken into consideration the variant patterns of NLPHL. There has been only one Indian study about variant patterns of NLPHL. This study published by Shet et al., in 2014 devised a three tier scoring system to quantify the variant patterns in a patient and help the treating physician in understanding the extent of variation.

We did a detailed study of the clinicopathologic features of 52 cases of NLPHL retrieved from our archives over a 10 year period.

CLINICAL FEATURES:

AGE:

The mean age at diagnosis of NLPHL was found to be 31 years, with a standard deviation of 14.181. The youngest patient in our study was 9 years and the oldest patient was 73 years old. The mean age at diagnosis was in keeping with published studies. (14)(15)(16) Patkar et al., in their analysis of 451 cases of Hodgkin lymphoma which included 54 cases (11.97%) of NLPHL from a predominantly Western Indian population, found the mean age at diagnosis to be 35 years. (21) In a recent population based analysis of 1162 cases of NLPHL done by Gerber et al., the median age at diagnosis was found to be 38 years. (55) According to large studies by Diehl et al., and Nogova et al., the average age at diagnosis was found to be between 30 and 40 years. (56)(29)

GENDER:

There was a marked male preponderance with 85.7% (42 of 49 cases) of the patients being male and only 14.3% (7 of 49 cases) being female. This was in keeping with the published data. (17)(21)

LYMPH NODE GROUP INVOLVED:

The most common lymph node group involved was found to be the cervical lymph nodes, followed by axillary and inguinal lymph nodes, in keeping with the published data. The other less commonly involved lymph node groups observed in our study

were pre auricular, supraclavicular, intra-abdominal and para-aortic lymph nodes in order of frequency. None of the cases had mediastinal involvement. The lymph node group involved was not known in 2 of our biopsies.

In our study, 41 of 49patients (83.67%) presented with single lymph node group involved, 1 of 49 (2.04%) patients with two lymph node groups involved on the same side of the diaphragm and 5 of 49 patients (10.20%) with two or more lymph node groups on both sides of diaphragm. In 2 patients the stage was not known. Five of the patients (10.2%) included in our study had definite bone marrow involvement.

According to literature, majority of the patients present with clinical Stage IA or IIA disease. (24)(57)(58) In a comprehensive analysis done by the German Hodgkin Study Group in 2008 which included 394 cases of LPHL, 63% of the patients were in early favourable stage (clinical stage I or II with no risk factors), 16% in early unfavourable stage (clinical stage I or II with risk factors) and 21% in advanced stage of the disease (clinical stages IIB with risk factors, III and IV). 28% of the LPHL cases had ≥ 3 nodal areas involved and 6% of their cases had extra nodal involvement (29)

In Shet et al's study, 32 of 80 patients (40%) had Stage I disease, 27 of 80 patients (33.75%) had Stage II disease, 13 of 80 patients (16.25%) had Stage III disease and 8 of 80 patients (10%) had Stage IV disease. (7)

According to Shankar et al., children usually present with peripheral lymphadenopathy and the inguino-femoral group of lymph nodes is less commonly involved. (24) Our study included 12 children and adolescents (<18 years of age) and the incidence of involvement of each lymph node group is as follows:

Cervical 33.4% (4 out of 12 biopsies), inguinal 25% (3 out of 12 biopsies), axillary 16.7% (2 out of 12 biopsies), preauricular, intra abdominal and para-aortic 8.3% each (1 out of 12 biopsies each).

DURATION OF SYMPTOMS:

The duration of symptoms in our study ranged from 1 month to 30 years. There has found to be a time lag between the first appearances of lymph nodes to the confirmation of NLPHL. (24)

CAPSULE:

Of the 52 biopsies included in our study, the capsule could be assessed in only 39 biopsies. Thickening of capsule was identified in 24 of 39 biopsies (61.5%) and the capsule was normal in the remaining 15 of 39 biopsies (38.5%). To the best of our knowledge, there has been no published data on capsular thickening in NLPHL.

EFFACEMENT OF ARCHITECTURE:

Effacement of architecture, whether complete/diffuse or partial was assessed in 49 of 52 biopsies, as 3 of the biopsies were core biopsies. There was complete effacement in 46 of 49 biopsies (94%) and partial effacement in only 3 of 49 biopsies (6%). Hence we conclude that lymph node involvement by NLPHL is more often associated with a complete effacement of architecture.

NODULAR AND DIFFUSE AREAS:

Nodularity was assessed in 50 of 52 biopsies because of the 3 core biopsies; two of them did not show nodular areas. 2 of 50 biopsies (4%) had ≤10% and 2 of 50

biopsies (4%) had 11-50% nodularity. The remaining 46 of 50 biopsies (92%) had >50% nodularity in the lymph nodes examined.

12 of 52 biopsies had diffuse areas and the percentage of diffuse areas ranged from 20% to 90% respectively. 8 of 12 biopsies (66.67%) had diffuse areas ranging from 11 to 50% within the nodes and 4 of 12 biopsies (33.33%) had >50% diffuse areas.

The significance of estimating the percentage of nodularity is important as there have been controversial reports with regards to the recurrence rates with nodular and diffuse histology.

In 1999, Ha et al., assessed the relapse rate in nodular and diffuse LPHD and found that in the nodular type, there were 9 of 16 relapses in 5 years and in the diffuse type 3 of 3 cases relapsed within 3 years itself. However, this was statistically insignificant. (59)

Earlier studies by Crennan et al., Borg-Grech et al., Tefferi et al., have shown no significant difference in the survival rate between the nodular and diffuse variants. (60)(61)(63)

Shet et al., stratified 72 patients into two groups, based on a scoring system which took percentage of nodularity as one of the 5 parameters to assess the relapse potential and management of NLPHL patients. The percentage of nodularity was graded as 100% nodularity-score 0, 75-100% nodularity-score 1 and <75% nodularity-score 2. Statistical analysis was done using ROC curve which showed that tumours with 77-85% nodularity had a lower chance for recurrence.

PERINODAL INFILTRATION:

Perinodal infiltration was assessed in 47 biopsies, of which only 1 biopsy (2%) had perinodal infiltration and the remaining 46 of 47 biopsies (98%) did not have any evidence of the same.

BACK TO BACK ARRANGEMENT OF NODULES:

46 of 50 biopsies had back to back arrangement of nodules and the remaining 4 of 50 biopsies did not have the same. One of the 3 core biopsies had focal back to back arrangement of nodules and the same could not be assessed in the other 2 core biopsies.

At least one nodule should be present within a lymph node to make a diagnosis of NLPHL. According to Fan et al., pattern A-D have nodular rich pattern and patterns E and F have diffuse patterns. In our study, 23 of 24 biopsies of Pattern A, 3 of 3 biopsies of Pattern B, 1 of 2 biopsies of Pattern C, 15 of 17 biopsies of Pattern D, 2 of 2 biopsies of Pattern E (in the nodular areas) and 2 of 3 biopsies of Pattern F had back to back arrangement of nodules.

SHAPE OF NODULES:

Of the 52 biopsies, nodules were present in 51 biopsies. 47 of 51 biopsies had round nodules only, 2 of 51 biopsies had serpiginous nodules only and 2 of 51 biopsies had both round and serpiginous nodules within the same lymph node.

The significance of identifying the shape of the nodules is that they help in the histomorphological characterisation of the disease into the variant patterns. Pattern A

shows prominent round nodules, Pattern B interconnected or serpiginous nodules, Pattern C ill defined nodules and Pattern F lacks distinct nodules. (2)

SMALL GERMINAL CENTERS:

Only 1 of 52 biopsies had small germinal centres within the nodules. In the analysis of 137 biopsies of 118 patients by Fan et al., 15% of the cases (17 of 118 patients) were found to have small germinal centres but they did not have any correlation with the clinical characteristics.(2)

SCLEROSIS:

Sclerosis was assessed in all 52 biopsies and was found in 32 of 52 biopsies (61.5%). 30 of these 32 biopsies (94%) had only focal sclerosis and the remaining 2 of 32 biopsies (6%) had extensive sclerosis. The types of sclerosis associated with NLPHL include: broad bands of sclerosis, extensive hyaline type of sclerosis and nodular masses of sclerosis.(2)

In a study done by the Stanford University on 137 biopsies of 118 patients, prominent sclerosis was found in 44% patients (12 of 27 patients) with disease recurrence and in 7% patients (2 of 29 patients) without recurrence. They showed that though prominent sclerosis appeared to be associated with disease recurrence, but was not an independent predictor of disease recurrence on multivariate analysis.(2)

GRANULOMAS:

Of the 52 biopsies of NLPHL included in our study, 5 biopsies (10%) had associated granulomas. Since histiocytes are one of the components of the reactive background in

NLPHL, they may form aggregates which resemble granulomas or may be seen scattered forming small granulomas at the periphery of the nodules. THRLBCL are usually not associated with granulomas. (10)(4)(24)

RESIDUAL NORMAL TISSUE WITH REACTIVE FOLLICLES:

7 of 52 biopsies (13%) had associated residual normal tissue with reactive follicles in the lymph node biopsy examined.

PROGRESSIVE TRANSFORMATION OF GERMINAL CENTRES:

None of the 52 biopsies included in our study had progressive transformation of germinal centres in the lymph node biopsies examined. There have been few studies that have shown an association between NLPHL and PTGC.(25)(26) However, Ferry et al., in 1992 followed up 5 patients with florid PTGC and proved that PTGC is not associated with increased risk for NLPHL. (28) Nguyen et al in their study found that three of eight patients with florid PTGC had recurrent/persistent lymphadenopathy and one of this patient developed NLPHD after 13 years. (64) In a recent study by Shet et al., 18 of 85 patients (21.18%) included in their study group had PTGC in subsequent or concurrent excisions. (7) Most of the patients do not develop Hodgkin lymphoma, however a close follow up of patients is required once a diagnosis of PTGC is made. (3)(64)(65)

OTHER CELLS:

The other cells which were assessed in our study include histiocytes, plasma cells and eosinophils. All 52 biopsies (100%) had histiocytes, 5 of 52 biopsies (10%) had rare plasma cells and 4 of 52 biopsies (8%) had rare eosinophils in the background.

RS LIKE CELLS:

In addition to the classical LP cells seen in 100% of cases, RS like cells were seen in only 36 of 52 biopsies (69%), of which 58% had only mononuclear RS like cells and 42% had both mononuclear and binuclear RS like cells.

IMMUNOHISTOCHEMISTRY:

VARIANT IMMUNOARCHITECTURAL PATTERNS:

In our study, we classified 51 biopsies of NLPHL into the 6 immunoarchitectural patterns as described by Fan et al. The biopsies were classified into the variant patterns based on the assessment of the H&E, CD20 and CD3 stained sections.

Classic nodular pattern (A) was seen in 25 biopsies, with 24 of these biopsies having it as their major pattern and 1 biopsy having a minor component of Pattern A.

Serpiginous/interconnected nodular pattern (B) was seen in 4 biopsies, of which one case had it as a minor pattern.

Nodular pattern with prominent extra nodular L&H cells (C) was seen in 2 biopsies.

Nodular pattern with T cell rich background (D) was seen in 19 biopsies with 2 of 19 biopsies having it as a minor pattern.

Diffuse pattern (T cell rich B cell lymphoma like) (E) was seen in 5 biopsies with 3 of 5 biopsies having it a minor pattern.

Only 3 biopsies had a moth eaten pattern with B cell rich background (F).

One patient had two biopsies from different lymph nodes with one node showing Pattern A, the other showing Pattern B.

A major finding in our study is that Pattern D was more frequently observed than what was observed by Fan et al, Hartmann et al and Churchill et al (Table.25).

These 6 immunophenotypic patterns were first described by Fan et al., in 2003 after evaluating 137 biopsies of 118 patients diagnosed as NLPHL over a time period of 6 years. A mixture of two or more patterns (hybrid patterns) was also seen in a good number of their cases (78 of 137 biopsies). Specific statistical analysis by the Classification and Regression Tree (CART) method showed that Pattern C was associated with a higher / early progression to a diffuse pattern. Their observation was that the mere presence of diffuse pattern (Pattern E) was associated with disease recurrence (P=0.00324) and when the diffuse pattern was the predominant pattern then it had a stronger chance of recurrence (P=0.00116). Hence, documentation of the presence of diffuse areas and their amount would be useful in the management of a patient. (2)

In 2013, Hartmann et al., assessed the frequency and prognostic implications of the variant patterns of NLPHL on 423 biopsies and classified them into tumour cell rich cases-abundant neoplastic cells focally forming sheets and not effacing the architecture (10 cases), typical NLPHL – patterns A and B (308 cases) and histopathologic variants – patterns C, D, E and F (105 cases).

Based on the histopathologic and clinical features, their patients were classified into 3 risk groups – high, intermediate and low. The PFS and OS were assessed. The 5 year PFS/OS for the low risk patients was found to be 95.2%/ 98.7%, for intermediate risk patients 87.5%/96.2% and for the high risk patients was 68.7%/88.3%. They found that the histopathologic variants were associated with poorer outcome, advanced disease and was found to be an independent risk factor for relapse. They also concluded that the higher risk group patients may be candidates for novel treatment strategies.(6)

Table.25: Comparison of the frequency of immunoarchitectural patterns between different studies

	A	**B**	**C**	**D**	**E**	**F**
Hartmann et al	80%	4%	7%	5%	2%	2%
Churchill et al	55%	20%	16%	2%	7%	
Fan et al	91%	0	0	7%	0	2%
Our study	47%	6%	4%	33%	4%	6%

Shet et al., selected 72 cases and quantified the variant patterns based on five parameters which included, 1) percentage of nodularity, 2) extranodular LP cells, 3) ratio of T cells vs B cells, 4) types of nodules and 5) loss of dendritic meshwork. They found that the patients with a lower score (≤ 6) had a better survival than those with a higher score (>6). The OS and DFS were also calculated. For patients with a score </=6, the 5 year OS was 100%, the median DFS was 133.6 months and 5 year DFS was 90%. For patients with a score >6, the OS was 87%, median DFS was 35months and 5 year DFS was 20%.

Thus the importance of characterising NLPHL into the variant patterns and their impact on prognosis has been highlighted by the above mentioned studies.

HYBRID PATTERNS:

Hybrid/mixed patterns were also observed in our study. Of the 51 biopsies included in our study, 44 biopsies had a pure/ single pattern and 7 biopsies had a mixture of two patterns. The hybrid/ mixed patterns observed were pattern D / E in 4 of 7 biopsies, pattern A / B in 2 of 7 biopsies and Pattern A / D in 1 biopsy. In our study most of the cases were of the single pattern and only a small number of cases had hybrid patterns which were in keeping with Hartmann et al's findings (Table.26).

We observed that all the hybrid Pattern E cases were associated with Pattern D, a finding that has not been documented so far. Thus we suggest that the nodular pattern with T cell rich background progresses to diffuse pattern (T cell rich B cell lymphoma like).

Table.26: Comparison of pattern distribution among various studies

	MAJOR PATTERN	2 PATTERNS	3 PATTERNS
Hartmann et al	331/413 (80.1%)	82/331 (19.9%)	6/413 (1.4%)
Churchill et al	33/67 (49.3%)	34/67 (50.7%)	
Fan et al	59/137 (43.1%)	35/137 (25.5%)	43/137 (31.4%)
Our study	44/51 (86.3%)	7/51 (13.7%)	

PROGRESSION TO DLBCL:

Of the 49 cases included in our study, progression to large B cell lymphoma / DLBCL was observed in 3 cases (6.12%). 2 of these 3 cases had a predominant pattern D and 1 case had pattern A in the NLPHL component examined. 2 of these 3 cases were in Stage 1 disease and 1 case was in Stage III disease.

NLPHL is an indolent disease and about 5% of cases progress to DLBCL. (12)(16)(38) Although earlier reports suggested that nearly one half of the cases of DLBCL following NLPHL are of the THRLBCL type (31), but the current literature states that NLPHL can progress to DLBCL (NOS) (3). Boudova et al., described two patterns in cases which were intermediate between NLPHL and THRLBCL1) - T/HRBCL like Pattern A and 2)DLBCL like Pattern B, the latter composed of tight clusters of large cells with a background of inflammatory cells and presenting with stage I or II disease. (66) In Fan et al's analysis of 118 patients, 8 of 56 patients with follow up (14.28%) progressed to DLBCL (which included conventional DLBCL,

DLBCL with T cell rich areas and THRLBCL. 5 of these 8 cases had diffuse areas prior to the onset of DLBCL. The reason of the higher incidence of DLBCL has been attributed to the referral bias in their study. However, they did not observe a correlation between the stage at presentation and progression to DLBCL. (2)

Shet et al., found transformation to large B cell lymphoma in 12 of 17 cases for who follow up biopsy was available. 11 of these 12 patients had THRLBCL like pattern and 1 of 12 patients had nodular DLBCL.(7)

There has found to be a clonal relationship between the neoplastic cells of NLPHL and DLBCL. There have been controversial reports on the course and prognosis of NLPHL progressing to DLBCL. Huang et al., found that the prognosis of NLPHL progressing to DLBCL and de novo DLBCL are the same and requires aggressive treatment. (67) However, majority of the studies and case reports suggest that DLBCL has a more indolent course similar to that of NLPHL when arising in such a setting. (68)(2)(69)(70)

RELAPSE/ RECURRENCE:

2 of 49 patients included in our study had a documented relapse. One patient initially presented with NLPHL-Pattern C and progressed to NLPHL-Pattern F after 2 years and the other patient progressed from NLPHL-Pattern A to NLPHL-Pattern D after 2 years. This progression to a higher pattern with diffuse areas was in keeping with Fan et al's observation. (2)

In Hartmann et al's effort to assess the prognostic implications of the variant patterns of NLPHL they found that 39 of 413 patients had progressed disease or a relapse within the first five years. The factors implicated as a significant cause/ risk factor for progression/relapse was found to be: variant pattern(C, D, E or F) (p=0.0038, OR=2.955), male sex (p=0.0119, OR=6.653) and low serum albumin (p=0.0055, OR=3.437).(6)

Shet et al., followed up 63 of 72 patients of NLPHL over a time span of 10 to 309 months and found that 20 patients (31.75%) had a relapse and 3 of them died due to progression to THRLBCL.(7)

ASSESSMENT OF SMALL AND LARGE B CELLS, INTRANODULAR AND EXTRANODULAR T AND B LYMPHOCYTES:

In our study, the T and B lymphocytes within and outside nodules were evaluated and quantified in the CD3 and CD20 stained sections (Table.27).

Table.27: Comparison of the median value of the B cells and T cells within and outside the nodules in each of the immunoarchitectural patterns of NLPHL

	A	B	C	D	E*	F
SMALL B CELLS						
Within nodules	80%	80%	85%	80%	80%	80%
Outside nodules	20%	20%	15%	20%	20%	20%
LARGE B CELLS						
Within nodules	92.5%	70%	30%	90%	50%	30%
Outside nodules	10%	30%	70%	20%	50%	70%
INTRANODULAR						
T lymphocytes	30%	40%	25%	80%	80%	70%
B lymphocytes	70%	60%	75%	20%	20%	30%
EXTRANODULAR						
T lymphocytes	80%	80%	75%	80%	70%	60%
B lymphocytes	20%	20%	25%	20%	30%	40%

* In nodular areas

Shet et al., quantified the extra nodular LP cells and graded them as 0-15%, 16-50% and >50% and the T cell rich areas as <20%, 21-50% and >50% with a score of 0, 1 and 2.These along with 3 other variables were scored and patients with a score of >6 had a higher stage of disease, median DFS of 35 months, 5 year DFS of 20% and 5 year OAS of 87%. This was in contrast to patients with a score of <6 who had a median DFS of 133.6 months, 5 year DFS of 90% and 5 year OAS of 100%. Thus

quantifying the above parameters would assist the pathologist to assign a pattern of NLPHL and thereby help the clinician in planning the treatment and follow up of the patient.

DIFFERENTIAL DIAGNOSES OF NLPHL:

The differential diagnosis of NLPHL includes THRLBCL, PTGC and LRCHL. Of these, THRLBCL is the most important because it has a significantly different prognosis. In our study we aimed to differentiate NLPHL from TCRLBCL using two novel immunohistochemical markers PD1 and CD57.

PD1 AND CD57 IN NLPHL:

In our study, which included 52 biopsies of NLPHL, immunohistochemical markers were done in 34 biopsies with sufficient tissue.

PD1 is a member of the CD28 receptor family which includes cytotoxic T lymphocyte associated antigen (CTLA-4), CD28, inducible co stimulator (ICOS) and B and T lymphocyte attenuator. It is expressed by the germinal centre associated T helper cells, inhibiting T cell activity. (51) It is expressed by the CD8 positive T cells and is associated with CD8 activation. There are at least two ligands for PD1 which include PD-L1 and PD-L2. In NLPHL, PD1 is expressed by the follicular T helper lymphocytes.

CD57, also known as Leu7, beta-1,3-glucuronyltransferase 1 and glucuronosyl transferase P, is a glycoprotein with cell adhesion functions. It is expressed in 57% of NLPHL cases.

BACKGROUND PD1 POSITIVE LYMPHOCYTES IN NLPHL:

Hartmann et al., studied the expression of PD1 along with CXCL13 and ICOS and found that T follicular helper cells (reactive background T cells) were higher in typical NLPHL (Pattern A and C) when compared to THRLBCL like NLPHL and THRLBCL. They also found that PD1 positive T cells (background T cells) were slightly more frequent than CXCL13 and ICOS in typical NLPHL, THRLBCL like NLPHL and THRLBCL (30% and 35% vs 20% and 15% vs 20% and 25% in patterns A&C; 15% vs 7.5% vs 5% in THRLBCL; 5% vs 5% vs 5% in THRLBCL). (36)

PD1 ROSETTES IN NLPHL:

PD1 rosettes are used in the diagnosis of NLPHL (71), especially to differentiate it from THRLBCL.

Hartmann et al., found that PD1 positive rosettes were frequent in typical NLPHL (8 of 10 cases of Pattern A and 6 of 10 cases of Pattern C) than THRLBCL like NLPHL (none of the cases had PD1 rosettes) and THRLBCL (none of the cases had PD1 rosettes). (36)

There have been only two studies till date that have assessed the usefulness of PD1 and CD57 rosettes in NLPHL and THRLBCL.(4)(5)

Nam Cha et al., in 2008 analysed the efficacy of PD1, CD57 and other immunomarkers in NLPHL and its differential diagnosis. PD1 rosettes were seen in 57 of 58 NLPHL cases (98.3%) which were in contrast to CD57 which was seen in only 44 of 58 NLPHL cases (75.9%). PD1 was found to be more sensitive than the other markers, including CD57. In NLPHL with diffuse areas (n=7), PD1 expression was seen in 5 of 7 cases (71.4%) and limited to the nodular areas, whereas CD57 expression was seen in all 7 cases and limited to the nodular areas. They also observed that the intensity of staining gradually reduced from the nodular to diffuse areas. In cases which were intermediate between NLPHL and THRLBCL, which included 5 cases, PD1 rosettes were seen in 4 of 5 cases (80%) in contrast to CD57 which was seen in 3 of 5 cases (60%). None of the THRLBCL cases (n=12) demonstrated PD1 rosettes. Thus they concluded that PD1 is a more sensitive marker and suggested it to be used as a routine immunomarker in NLPHL. (4)

Churchill et al., in 2010 compared PD1 and CD57 in the 6 patterns of NLPHL (n=67), de novo THRLBCL (n=6) and nodular LRCHL (n=5). Their results were in keeping with Nam Cha et al's findings that PD1 is more superior to CD57 in the nodular variants of NLPHL-A, B and D. (PD1 rosettes were seen in 87% of nodular NLPHL vs C57 rosettes seen in 50% of nodular NLPHL). Also the PD1 reactivity in Pattern C was present in 66% of cases when compared to 33% of cases with CD57. Another finding in their study was that there was gradual loss of expression of PD 1 from the nodular (56%) to diffuse (19%) areas.(5)

Since PD1 rosettes were present in THRLBCL cases also [2 of 6 cases (33%) with frequent PD1 rosettes, 1 of 6 cases (17%) with infrequent PD1 rosettes], they

mentioned that the loss of PD1 does not correlate with progression to DLBCL/THRLBCL.(5)

PD1 AND CD57 IN NLPHL IN THE PRESENT STUDY:

In our study, 25 of 32 biopsies (78.13%) showed frequent PD1 rosettes encircling the neoplastic large B cells, 1 of 32 biopsies (3.12%) showed infrequent PD1 rosettes and 6 of 32 biopsies (18.75%) did not have any PD1 rosettes in the presence of positive internal and external controls. PD1 did not work in 2 of the 34 biopsies.

19 of 34 biopsies (55.9%) showed frequent CD57 rosettes, 7 of 34 biopsies (20.6%) showed infrequent CD57 rosettes and 8 of 34 biopsies (23.5%) did not have any CD57 rosettes in the presence of positive internal and external controls.

Overall, PD1 staining was found to be more superior to CD57 in the diagnosis of NLPHL. All 6 biopsies which showed absent staining for PD1 also showed absent staining for CD57.

PD1 AND CD57 IN THRLBCL IN THE PRESENT STUDY:

PD1 rosettes were seen in only 1 of 10 cases (10%) and were infrequent. The remaining 9 of 10 cases showed absent staining for PD1 in the presence of positive internal and external controls.

CD57 rosettes were seen infrequently in 2 of 10 cases (20%) and were absent in the remaining cases (80%).

26 / 32 cases of NLPHL showed PD1 rosettes in contrast to 1 / 10 cases of THRLBCL. The sensitivity of PD1 was found to be 81.3% (95% confidence interval CI: 63.6%-92.8%) and specificity was 90% (95% CI: 55.5%-99.7%).

The positive likelihood ratio of PD1 is 8.13(95%CI: 1.26-52.6), i.e., if PD1 rosettes are present, there is 8.13 times more likely chance that the patient has NLPHL rather than THRLBCL. The positive predictive value of PD1 in the diagnosis of NLPHL was found to be 96.3%, i.e., 96.3% of patients who have PD1 positive rosettes actually have NLPHL rather than THRLBCL and the negative predictive value of PD1 was found to be 60%, i.e., 60% of the patients in whom PD1 rosettes are absent do not have NLPHL.

26 / 34 cases of NLPHL showed CD57 rosettes in contrast to 2 / 10 cases of THRLBCL. The sensitivity of CD57 was found to be 76.5% (95%CI: 44.4%-97.5%). The positive likelihood ratio of CD57 is 3.82 (95%CI: 1.09-13.4), i.e., if CD57 rosettes are present, there is only 3.82 times more likely chance that the patient has NLPHL rather than THRLBCL. The positive predictive value of CD57 in the diagnosis of NLPHL was found to be 92.9%, i.e., 92.9% of patients who have CD57 positive rosettes actually have NLPHL rather than THRLBCL and the negative predictive value of CD57 was found to be 50%, i.e., 50% of the patients in whom CD57 rosettes are absent do not have NLPHL. Table.28 compares the PD1 and CD57 rosettes in various studies including our study.

Table.28: Comparison of PD1 and CD57 rosettes in NLPHL and THRLBCL in different studies

	NLPHL		THRLBCL	
	PD1 ROSETTES	**CD57 ROSETTES**	**PD1 ROSETTES**	**CD57 ROSETTES**
Nam Cha et al	98.3% (57/58)	75.9% (44/58)	0 (0/12)	0 (0/12)
Churchill et al	82% (55/67)	45% (30/67)	50% (3/6)	0 (0/6)
Our study	81.25% (26/32)	76.47% (26/34)	10% (1/10)	20% (2/10)

The percentage of PD1 positive rosettes found in our study are comparable to Churchill et al's findings (81.25% vs 82%). 10% of our THRLBCL cases had PD1 rosettes which is much lesser in comparison to Churchill et al's findings.

Thus, we conclude that PD1 is a more sensitive and specific marker than CD57 in the diagnosis of NLPHL.

CONCLUSIONS

CONCLUSIONS

1. The mean age at diagnosis of NLPHL was found to be 31 years which is similar to Indian as well as International data.
2. There is a marked male preponderance in NLPHL with the male: female ratio being 6.4:1.
3. 51 biopsies of NLPHL diagnosed in our Institution between January 2003 and December 2013 was sub classified into 6 immunoarchitectural patterns. Pattern A was found to be the most common pattern (47.1%), similar to other studies. This was followed by Pattern D (33.3%). The incidence of Patterns B and F were 5.9% each and the incidence of Patterns C and E were 3.9% each.
4. Hybrid pattern was seen in 7 biopsies of NLPHL, of which Pattern D / E was the most common (8%).
5. All hybrid Pattern E cases were associated with Pattern D, hence we suggest that the nodular pattern with T cell rich background progresses to diffuse pattern (T cell rich B cell lymphoma like).
6. Transformation to diffuse large B cell lymphoma (NOS) was seen in 3 cases (6.12%) and Pattern D was the more common pattern found to be associated with DLBCL transformation.
7. Two patients had a documented relapse, one with progression from Nodular pattern with prominent extra nodular L&H cells (Pattern C) to a moth eaten pattern with B cell rich background (Pattern F), in keeping with Fan et al's findings and

the other with progression from Classic nodular pattern (Pattern A) to nodular pattern with T cell rich background (Pattern D), both after a period of two years.

8. Frequent PD1 rosettes were seen in Classic nodular pattern (93%) followed by the T cell rich nodular pattern (73%). All cases of Serpiginous/interconnected nodular pattern and Nodular pattern with prominent extra nodular L&H cells had frequent PD1 rosettes. Infrequent PD1 rosettes were seen in only moth eaten pattern with B cell rich background. Thus the nodular patterns had a higher frequency of staining with PD1 than the diffuse variants.
9. Frequent CD57 rosettes were seen in the Nodular pattern with prominent extra nodular L&H cells (100%), Classic nodular pattern (80%), T cell rich nodular pattern (42%) and Serpiginous/interconnected nodular pattern (33%). Patterns D, A and B showed infrequent CD57 rosettes also. Diffuse pattern (T cell rich B cell lymphoma like) and moth eaten pattern with B cell rich background lacked CD57 rosettes in all cases.
10. THRLBCL showed only infrequent rosettes with both PD1 (10%) and CD57 (20%).
11. The sensitivity of PD1 was found to be 81.3% (confidence interval-95% CI: 63.6%-92.8%) and specificity was 90% (95% CI: 55.5%-99.7%). The sensitivity of CD57 was found to be 76.5% (95% CI: 44.4%-97.5%) and specificity was 80% (95% CI: 44.4%-97.5%). Thus PD1 is a more sensitive and specific marker of NLPHL.

LIMITATIONS

LIMITATIONS

1. Our sample size was relatively small though our study period was 10 years.
2. The efficacy of the immunohistochemical markers PD-1 and CD57 could be assessed only on 34 biopsies of NLPHL due to the lack of tissue. Immunohistochemical markers could not be done on few cases which were received as consultation.
3. Since this was a retrospective study, follow up details of patients and prognosis of the patients could not be assessed and correlated with the immunoarchitectural patterns.
4. Only 2 cases of Pattern E [Diffuse pattern (T cell rich B cell lymphoma like)] were present in our study, of which only one had sufficient tissue to perform immunohistochemical markers. This case lacked PD1 and CD57 rosettes and hence the efficacy of PD1 and CD57 in this pattern could not be assessed.

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ANNEXURE

ANNEXURE

Appendix 1: PROTOCOL FOLLOWED FOR AUTOMATED IMMUNOSTAINING OF PD1 AND CD57

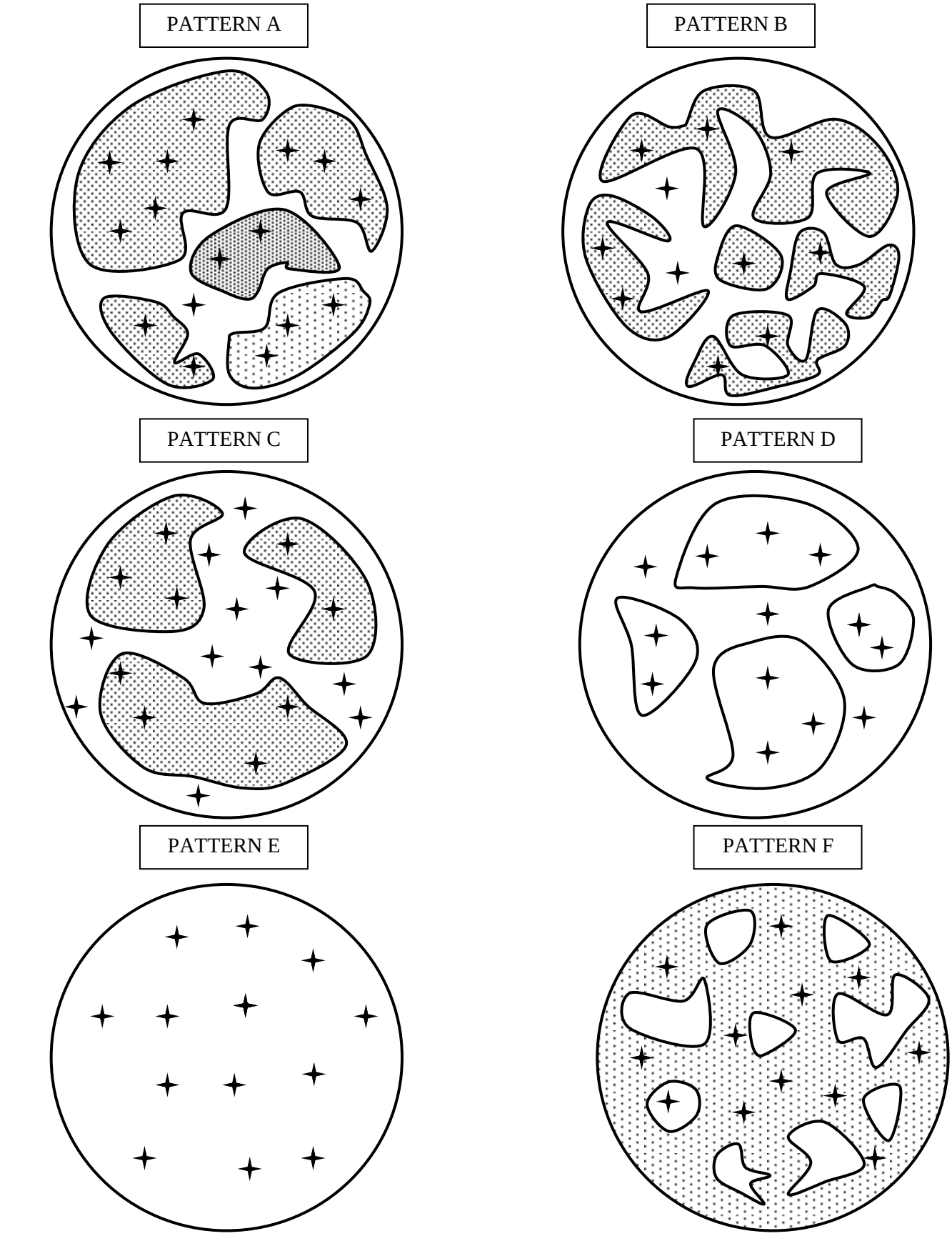
1. Paraffin embedded tissue sections were cut at 3µ thickness and floated in poly L-lysine coated slides and incubated overnight at 37˚C.
2. These slides were then treated with 4% milk solution for 10 minutes to eliminate the hydrophobic effect and give positive charge to the slides.
3. Then the slide labels were bar coded and the labeled slides were loaded in Ventana Benchmark XT autostainer (a fully automated immunostainer).
4. Individual protocols have been designed in the software attached to the machine for each marker. Specific protocols were selected according to the marker.
5. A standard protocol was used for both the markers included in our study. The steps included in this protocol were as follows:
    a. Deparaffinization
    b. Liquid cover slip application.
    c. Heat induced antigen retrieval by treating with standard CC1 solution (pH patent with the company) for one hour at 90˚C.
    d. Then the primary antibody was added and incubated for 40 minutes @ 37˚C.
    e. Then the secondary antibody (Multimer) was added and incubated for 8 minutes.

f. Finally the slides were counterstained with Haematoxylin and incubated for 8 minutes, followed by incubation with the bluing reagent for 4 minutes.

   (From antigen retrieval till counterstaining, in between every step the slides were washed with reaction buffer. The whole process is automated).

g. Then the slides were brought to 80% alcohol (2 changes) to remove the liquid cover slip and then dried and mounted in DPX.

Appendix.2: VARIANT IMMUNOARCHITECTURAL PATTERNS OF NLPHL (ADAPTED FROM FAN ET AL) (2)



Appendix.3: NLPHL PROFORMA

Serial no: Biopsy no:

Age: Gender: M/F

CLINICAL:

LN group involved:

Duration of symptoms:

B symptoms: +/-

GROSS:

LN size:

Cut surface: colour consistency

MICRO:

Capsule: Thickened/ normal

Effacement of architecture: Partial/ complete

Nodularity (%): %

Perinodal infiltration: + / -

Back to back arrangement of nodules: +/ -

Shape of nodules: Round/ serpiginous

Diffuse areas: +/ - If positive: %

Small germinal centres: +/ -

Sclerosis: +/ - If +, focal / extensive

Granulomas: +/ -

LP cells:

Within nodules: +/ -

Outside nodules: +/ -

RS like cells:

Within nodules: +/ -

Outside nodules: +/ -

Mononuclear / binuclear

Perinucleolar halo: +/ -

Inclusion like nucleoli: +/ -

Residual normal tissue with reactive follicles: +/ -

PTGC: +/ -

Other cells: Histiocytes / plasma cells / eosinophils

IHC:

LP cells: CD15 +/ - CD30 +/ -

CD 15: % CD30: %

Membrane stain / Golgi stain:

Small B cells (CD20): Large B cells (CD20):

Within nodules: % Within nodules: %

Outside nodules: % Outside nodules: %

Intranodular lymphocytes: Extranodular lymphocytes:

T lymphocytes: % T lymphocytes: %

B lymphocytes: % B lymphocytes: %

T cell rosettes: %

Oct 2 in large cells: +/ -

Bob 1 in large cells: +/ -

Other markers:

PD-1 rosettes: frequent / infrequent / absent

Background cells PD 1: +/ -

CD 57: frequent / infrequent / absent

Background cells CD 57: +/ -

NLPHL pattern: A/B/C/D/E/F

Major pattern: %

Minor pattern: %

Appendix 4: THRLBCL PROFORMA

Serial no: Biopsy no:

Age: Gender: M/F

CLINICAL:

LN group involved:

Duration of symptoms:

B symptoms: +/ -

GROSS:

LN size:

Cut surface: colour consistency

MICRO:

Capsule: Thickened/ normal

Effacement of architecture: Partial/ complete

Nodularity: + / - , If +, %

Perinodal infiltration: +/ -

Small germinal centres: + / -

Sclerosis: +/ - If +, focal / extensive

Residual normal tissue with reactive follicles: +/ -

Prominent blood vessels: +/ -

Necrosis: +/ - If +, %

RS like cells: +/ -

Mononuclear / binuclear

Perinucleolar halo: +/ -

Inclusion like nucleoli: +/ -

Other cells: Histiocytes / plasma cells / eosinophils

IHC:

CD20 positive B-cells: % T cell rosettes: %

PD-1 rosettes: frequent / infrequent / absent

Background cells PD 1: + / -

CD 57: frequent / infrequent / absent

Background cells CD 57: + / -